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A Complement-Fixation Test for Identification and Differentiation of Immunological Types of the Virus of Vesicular Exanthema of Swine

R. A. BANKOWSKI, D.V.M., Ph.D.; R. WICHMANN, B.S.; M. KUMMER, B.A.

Davis, California

THE RAPID spread of vesicular exanthema (v.e.) of swine from California, in which it was enzoötic for twenty years, to hog populations of 40 states during the past ten months has placed this infection in a class of disease which is potentially as contagious among susceptible livestock as foot-and-mouth disease. Since a clinical differentiation of the vesicular diseases among the domestic animals is impossible and present methods of animal inoculation are time consuming and costly, a rapid laboratory procedure which would aid in arriving at a diagnosis is of great importance to the veterinary profession and the livestock industry.

This report deals with a complement-fixation test (c.f.) which apparently is capable of identifying and differentiating the immunological types of v.e. within twenty-four hours. The test is performed with vesicular material collected from infected hogs in the field. Such material has been collected and submitted to this laboratory since April, 1951, by veterinary personnel of the official regulatory agencies within the State of California. Without such cooperation the development of this test would have been delayed.

MATERIALS AND METHODS

Viruses.—During the course of this study, three immunologically and serologically distinct types of v.e. virus have been encountered.

Type F, v.e. virus, was obtained by Dr. J. Traum in 1948 from infected hogs in the vicinity of Fontana, Calif. The virus was stored at low temperatures * until its first passage through swine in the laboratory in the fall of 1950. Antiserums against this type of virus have been produced, using pooled tissues containing the virus as antigen obtained from hogs in the second through the fourth serial passages. These passages were necessary since the virulence of the virus during these periods appeared relatively mild.

Type B, v.e. virus, was isolated in September, 1951, from an outbreak which occurred in a group of 90 grain-fed hogs in the vicinity of Davis. Beginning with the first passage in the laboratory, a severe type of the disease has been produced in swine with regularity.

Type A, v.e. virus, was isolated in September, 1952. It was obtained from an infected herd of garbage-fed hogs in the vicinity of San Francisco.

The v.e. viruses were differentiated from foot-and-mouth disease and vesicular stomatitis by injection of each into various species of domestic animals but differentiation of the immunological types of v.e. from one another was performed by cross-immunity tests in swine.[1, 2]

Diluent.—Freshly prepared 0.9 per cent saline solution from chemically pure NaCl was used in all titrations and in the test unless specified otherwise.

Sheep Cells.—Blood of sheep was obtained in the usual manner and after washing three times with 0.9 per cent saline solution was stored at 5 C. under Alsever's solution. For the test, a sufficient amount of cells was washed and a 3 per cent red blood cell suspension prepared, which was finally adjusted with the aid of the Coleman, Jr., spectrophotelometer in 12-mm. absorption tubes to an optical density of 0.34 by the method described by Mayer, *et al.*[3]

Sensitized Sheep Cells.—Sheep red blood cell rabbit antiserum which was prepared in this lab-

From the School of Veterinary Medicine, University of California, Davis.

Project supported in part by grants from the U. S. Bureau of Animal Industry, U.S.D.A., and the Public Health Service of the National Institute of Health.

* Movement of the laboratory from Berkeley to Davis necessitated transfer of the virus to various low temperature refrigerators during the storage period; however, every effort was made to maintain the virus between 0 C. and −15 C.

oratory was employed. Titration of hemolysin was made in 0.25-ml. volumes of doubling dilutions beginning with primary dilutions of 1 : 100 and 1 : 150, respectively. The titrations were completed by adding 0.5 ml. of saline and 0.25 ml. of each of complement, and sheep cells. Readings were made after thirty minutes' incubation at 37 C. to determine the hemolytic unit. A dilution of amboceptor containing four hemolytic units was prepared and poured into an equal volume of sheep cells and the mixture placed at 37 C. for fifteen minutes to form the sensitized sheep cell suspension used in the test.

Complement.—The serums of at least 12 guinea pigs were used as a source of complement. The animals were starved for twenty-four hours, exsanguinated by severing the large blood vessels of the neck, and the blood allowed to clot in sterile Petri dishes at room temperature. For convenience, the pooled and centrifuged serum was dispensed in 1- or 2-ml. amounts in rubber-stoppered Wasserman tubes or hermetically sealed ampules and stored at −15 F.

Because of the procomplementary activity of swine serum, the titration of complement was performed in the presence of 0.25 ml. of normal swine serum and each antiserum employed in the test. The complement was diluted and added to the serum in volumes ranging from 0.05 ml. to 0.5 ml. at step intervals of 0.05 ml. Cold 0.9 per cent saline solution was added to each tube to bring the volume to 0.75 ml. A 0.5-ml. quantity of sensitized sheep cells was added and the mixtures incubated at 37 C. for thirty minutes. A full unit of complement used in the test was designated as 0.05 ml. in excess of the amount of guinea pig complement in the presence of 0.25 ml. of swine serum which caused complete and sparkling hemolysis.

Antigen.—The antigen for the test was prepared from the coverings of unruptured vesicles of the snout or feet of infected swine. Lesions produced by intradermal inoculation into the snouts of pigs with laboratory strains of v.e. virus develop in twelve to eighteen hours. Only fresh, unruptured vesicles from hogs with high fever were harvested because materials collected after the peak of development of the vesicles were found to produce an unsatisfactory antigen. Similar observations have been reported with vesicular stomatitis virus by Wagener.[4] The vesicular coverings were collected into glycerine phosphate buffer solution (pH 7.4) and stored at −15 F.

Two of the greatest difficulties encountered with field samples submitted for testing at the beginning of the program were (1) they contained a considerable amount of hair and debris and (2) the collection of insufficient amounts of material for the test. Vesicular material collected for the laboratory should be removed from hogs in the acute stages of the disease, and each specimen should be accompanied by a description of the lesions and the body temperatures of the animals from which the material was removed.

The antigens for the test were prepared as follows: Pieces of vesicular coverings were washed with cold, sterile, buffered saline solution (pH 7.4), weighed and transferred to a cold, sterile mortar. A sufficient amount of sterile alundum and a measured quantity of buffered saline (Ca, 2 ml.) were added and the mixture thoroughly ground. When the tissues were reduced to a finely divided mass, the suspension was transferred to a pyrex round-bottom 40-ml. centrifuge tube and the residue in the mortar washed into the tube with saline to make a 10 per cent suspension. The latter was centrifuged at 3,000 r.p.m. (1,600 g.) for twenty minutes and the slightly opalescent supernatant fluid was used as a crude antigen. Such antigens were usually found to be anticomplementary. Recentrifugation at higher speeds may remove this undesirable activity; however, the added step did not give consistent results. Studies of methods of purifying the antigen and rendering them complementary by ether extraction[5, 6] and by treatment with Lloyd's reagent[5] showed the latter method was superior. The procedure is simple, rapid and no serious anticomplimentary activity was noted in virus suspensions prepared from over 200 samples treated by this method. The standard procedure adopted consists of adding 0.5 to 1.0 Gm. of Lloyd's reagent to 10 ml. of crude antigen, shaking the mixture, and allowing it to stand in the refrigerator for fifteen minutes. The suspension is then centrifuged at 2,500 r.p.m. (1,200 g.) for ten minutes and the clear, supernatant fluid is ready for use.

Antigens were prepared immediately before testing or were prepared in sufficient numbers on one day and held at 4 C. for testing the following day. The latter method was adopted as a routine procedure during v.e. outbreaks when large numbers of samples were submitted.

Antiserums.—Hyperimmune antiserums against the three types of v.e. virus were prepared in both swine and guinea pigs. Serums of swine were preferable since they are easily produced and heterologous fixation antibodies, due to foreign proteins in the inoculums for the production of antiserums, are unlikely to occur.

Although swine antiserums are readily available and will fix complement in the presence of homologous antigens, it is unique in that it is procomplementary, which may be due to the high concentration of heat-stable components of complement.[7] It is important, therefore, that each antiserum used in the test be included in the guinea pig complement titration to determine the true complementary activity.[8] High titered antiserums have been produced in swine by inducing the disease with the desired virus by intradermal

inoculations into the snout. The unruptured vesicles of the snout and the feet were harvested and a 5 per cent suspension was made of the pooled material which was stored at —78 C. for use during the hyperimmunization procedure. When the swine recovered and all lesions were healed, 2.0-ml. quantities of the homologous virus suspension were injected subcutaneously at four-day intervals for at least four times. For the production of guinea pig serums, 24 animals were usually placed within the same unit of the isolation quarters and inoculated intradermally into the tarsal pads with 0.05- to 0.1-ml. quantities. The schedule of hyperimmunization of guinea pigs was identical to that employed for swine but the injections consisted of 0.25 ml. of the suspension intramuscularly. All of the animals were bled four to eight days following the last hyperimmunizing dose and the serums from each species pooled. No preservative was added but stock hyperimmune serums were stored at —6 C.

Titration of Vesicular Exanthema Antiserums.—Because of the cross fixation which occurs with any of the three types of v.e. viruses with heterologous hyperimmune serums, it was found necessary to titrate each serum with its corresponding antigen to determine the maximum amount of hyperimmune serum which specifically reacted with its homologous virus without cross fixation with any of the other types of v.e. virus. This reaction is depicted by the results shown in figure 1 in which serial dilutions of type A antigen were tested against decreasing dilutions of its strain homologous antiserum and the two other antiserums prepared with immunologically distinct types of virus (types B and F). It is evident from this graph that a 2 or 4 per cent type A antiserum would fix its homologue, and cross fixations would be inapparent with the other two types.

These titrations were conducted with freshly harvested material removed from hogs at the height of vesicular formation under controlled conditions in which excellent fixation reactions may be expected. On the other hand, vesicular material from

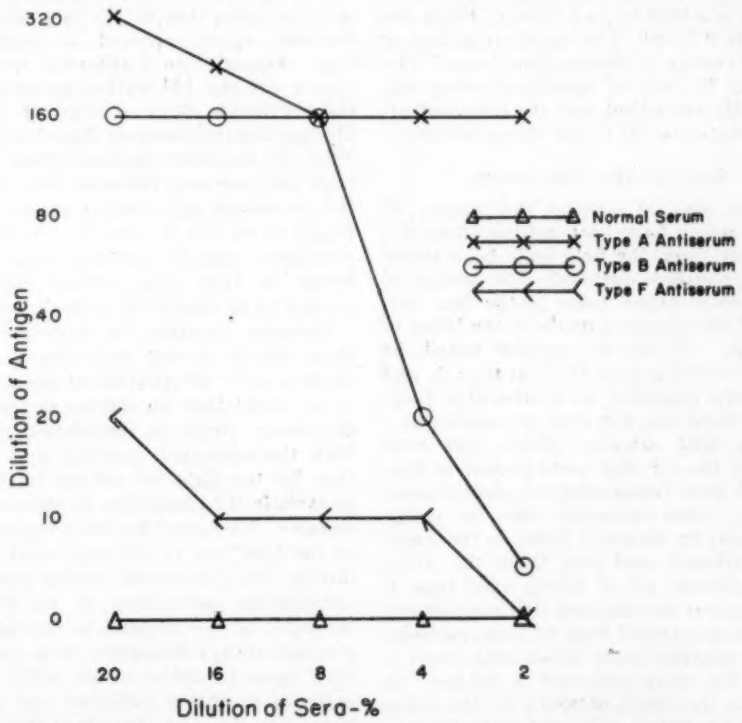

Fig. 1—Effect of dilution of antiserums upon cross fixation with type A vesicular exanthema virus.

the field can not be as carefully selected and, as the samples are at least ten days old before they are submitted to the laboratory, fixation to such high titers is not the rule. Fixation titers of antigens prepared from field samples generally range from 1 : 10 to 1 : 40 but on occasions may reach 1 : 80.

The Test.—The technique in general consists of placing 0.25-ml. amounts of previously titrated hyperimmune serum and 1 full unit of complement, as previously determined in the presence of swine antiserums, into each of five tubes containing 0.25 ml. of antigen, serially diluted 1 : 10, 1 : 20, 1 : 40, 1 : 80, and 1 : 160. Since each antigen is titrated against swine hyperimmune serums of all three immunologically distinct types of v.e. virus and a normal swine serum, the amount of guinea pig complement added to the serially diluted antigen and serum mixtures may vary depending upon the procomplementary activity of the swine serums employed. Saline (0.9 %) is added to each tube to bring the volume to 0.75 ml. The racks are placed at 4 C. for twenty to twenty-four hours. The next day 0.5 ml. of sensitized sheep red blood cells are added and the test read after incubation at 37 C. for thirty minutes.

RESULTS AND DISCUSSION

At the time of writing this paper, 83 samples which have been submitted to this laboratory from the field have been tested by the procedure outlined. The testing of field samples began prior to the first outbreak of the disease outside of the State of California. Of the 83 samples tested, 58 were identified as type B, 17 as type A, and eight were classified as undesirable tests. Type F virus has not been encountered.

Seven field samples which had been typed by the c.f. test were picked at random and their immunological identification made by cross-immunity tests in swine. Three samples obtained prior to the June, 1952, outbreak and two from the June, 1952, outbreak, all of which fixed type B hyperimmune serums, and two samples collected from infected hogs on local garbage-feeding establishments which conformed to type A, v.e., were subjected to the test. In each case, the result obtained by the cross-immunity tests was in accord with the serological differentiation.

Tissues of the first outbreak tested by the c.f. test were harvested from swine which were found infected in a local garbage-feeding establishment on April 9, 1951, and the results showed that it was caused by type B, v.e. virus. Samples from nine additional outbreaks occurring in garbage-feeding establishments in the northern part of the state prior to June 24, 1952, in all cases proved to be type B. Type B was also incriminated as the causative agent in the June, 1952, outbreak as determined by the cross-immunity tests in swine and by the c.f. tests performed on material harvested from lesions of pigs found infected upon entry into the state. The last reported infection with v.e. in imported hogs in this outbreak was Aug. 12, 1952.

In the early part of September, 1952, another type of v.e. virus was suspected as involving local garbage-fed hogs and was identified by cross-immunity tests in swine, and later by the c.f. test, as type A. This virus was consistently identified in field samples submitted to the laboratory until v.e. was again reported as occurring in hogs shipped into California from other states. Of the 144 outbreaks recorded by the California State Bureau of Livestock Disease Control between Jan. 9 and Feb. 8, 1953, 45 samples obtained from infected hogs shipped into the state and tested by this procedure all, without exception, were found to be due to type B, v.e. virus. An occasional sample obtained from an outbreak in local hogs during this period proved to be caused by type A, v.e. virus.

Samples classified as undesirable were those which showed less than a complete fixation at 1 : 10 dilution of antigen. It is to be noted that no sample is sent to the laboratory prior to inoculation of calves with the suspected material and observation for ten days by official veterinarians to exclude the possibility of other vesicular diseases. No record has been maintained as to the handling of the harvested material during this differential testing period, and information pertaining to handling and shipment of the samples to the laboratory was not always available. It is possible in such cases to infect swine with the field material to obtain sufficient and adequate virus for the test, but facilities for this type of experimentation were limited.

SUMMARY

A complement-fixation test for the identification and differentiation of three immunologically distinct types of the virus inducing vesicular exanthema in swine has been described. The test employs hyperimmune swine serums and vesicular material harvested from infected hogs in the field.

References

[1] Traum, J.: Foot-and-Mouth Disease: Specific Treatment, Eradication, and Differential Diagnosis. Proc. Twelfth Internat. Vet. Cong., *2*, (1934): 87.

[2] Bankowski, R. A.: Some Factors in the Identification of the Vesicular Viruses. California Vet., *6*, (1952): 20.

[3] Mayer, M. M., Eaton, B. B., and Heidelberger, M.: Spectrophotometric Standardization of Complement for Fixation Tests. J. Immunol., *53*, (1946): 31.

[4] Wagener, K.: Investigations on the Virulence of Vesicular Stomatitis Virus and the Properties of Immune Serums. J.A.V.M.A., *81*, (1932): 160.

[5] Palacios, R., and Rodriguez, R.: Deviation of the Complement-Fixation Test in the Study of Foot-and-Mouth Disease. Bull. Off. Internat. Epizoöt., *31*, (1949): 75.

[6] Camargo, F., Eichhorn, E. A., Levine, J. M., and Telles-Giron, A.: A Complement-Fixation Technique for Foot-and-Mouth Disease and Vesicular Stomatitis. Proceedings Book, AVMA, (1950): 207.

[7] Jonsen, J., Manski, W., and Heidelberger, M.: Pig Complement and Its Fractionation for the Production of Reagents Containing Third Component. J. Immunol., *67*, (1951): 385.

[8] Bankowski, R. A., Wichmann, R., and Kummer, M.: Procomplementary Activity of Swine Sera. In preparation.

A Study of the Effect of Repeated Intraperitoneal Injections of Glucose in Pigs

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Urbana, Illinois

THE NORMAL gastric emptying time in the pig is prolonged, requiring a fast of many hours to completely empty the stomach. As a result, digestion and absorption are almost continuous processes. This may have a bearing on the belief that the pig has an unusual carbohydrate tolerance when a rapidly absorbable form of sugar is given by mouth. Lukens [18] found that pancreatectomy produced only a mild form of diabetes in the pig. This suggests that the endocrine system of the pig as related to carbohydrate metabolism differs from some other animals, especially the dog.

LITERATURE CITED

Homans [16] reported that removal of approximately three-fourths of the pancreas in the cat resulted in hyperglycemia followed by degeneration of the *beta* cells of the islets of Langerhans in the remaining pancreas. By removing portions of the pancreas from dogs, Allen [1] succeeded in producing hyperglycemia with degeneration of the *beta* cells. If hyperglycemia persisted for four to seven days, degeneration of the *beta* cells occurred.

Jacobs and Colwell [17] found that the continuous intravenous administration of glucose to dogs proved fatal. No specific lesions were observed in the islets of Langerhans. Woerner [20] gave continuous intravenous injections of glucose to guinea pigs. Hyperglycemia was pronounced but examination of the islet cells did not reveal any change in the *beta* cells.

Dohan and Lukens [10] made extensive observations on cats which were given glucose by intraperitoneal injection. Approximately, the splenic half of the pancreas was removed surgically from 8 of the 35 cats used in this study. Ten of the cats had blood glucose concentrations of 149 mg./100 ml. for nine or more days and developed hydropic degeneration of the islet cells.

In 1893, Minkowski [19] reported the results of partial pancreatectomy in 1 pig. He noted that the operation did not produce the severe diabetes typical of the dog. Gould and Carlson [12] depancreatized 2 pigs. They likewise observed that the resulting diabetes was mild. Later, they performed pancreatectomy on 2 pigs and reported some of the metabolic results. One pig lived sixty-six days, although no pancreas was found at autopsy.

Lukens [18] depancreatized 12 pigs. Subsequent autopsy examination revealed that 9 were completely depancreatized. These pigs became glycosuric when fed, but the fasting glucose excretion was slight or absent. Ketonuria was mild and there was no acidosis.

He compared the depancreatized diabetic pig with findings in the hypophysectomized or adrenalectomized diabetic dog and cat. He suggested that the seemingly mild diabetes of the pig is due to the nature of its pituitary (or other endocrine) function rather than to the utilization of carbohydrate in the absence of insulin.

Hanawalt, Link, and Sampson [15] concluded that the form of the carbohydrate tolerance curve obtained after the administration of glucose to normal pigs by the intravenous route apparently closely parallels the type of curve procured when the test is made on normal human subjects.

This study was undertaken to observe the effects repeated intraperitoneal injections of glucose had on various body processes in the pig.

EXPERIMENTAL PROCEDURES

Animals.—Twenty apparently healthy pigs of both sexes ranging in weight from 18 to 66 lb. were used. They were maintained in metabolism cages for the major part of each experiment to allow collection of urine. A liberal amount of a balanced ration was fed. In addition, intramuscular injections of vitamin B complex were given to aid in maintaining their appetites during the injections periods. Water was provided *ad libitum*.

Injections.—The standard solution for intraperitoneal injection contained 20 per cent glucose and 0.75 per cent sodium chloride. It was found that more concentrated solutions caused adhesions to develop in the peritoneal cavity. Injections were made each morning and evening at approximately ten- and fourteen-hour intervals. The condition of the animal, and the levels of glycemia and glycosuria were used as indications when to terminate injections.

Collection of Specimens.—To avoid change in composition, R.P.C.* preservative tablets were

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* Potassium acid phosphate 0.100 Gm.
Sodium benzoate 0.050 Gm.
Benzoic acid 0.065 Gm.
Urotropin 0.050 Gm.
Sodium bicarbonate 0.010 Gm.
Mercuric oxide, red 0.001 Gm.

placed in the urine collection bottles. The urine was collected twice daily and the volume recorded.

Blood samples were collected from the anterior vena cava by the technique of Carle and Dewhirst.[6] Sodium fluoride was used as an anticoagulant. The majority of observations were made on samples taken just before the morning injection and so represented the lowest value in the 24-hour period. When injections were discontinued, blood samples were collected before the morning feeding. Liver tissue collected at autopsy for glycogen determination was placed immediately in 30 per cent potassium hydroxide.

Chemical.—Blood glucose determinations were made by the Somogyi[25] method, using Nelson's[21] color-producing reagent with readings on an Evelyn photometer. Blood ketones were determined gravimetrically by the Van Slyke and Fitz[27] method. Cholesteral was determined according to the Bloor[7] method.

The Benedict[4] method was used for urine glucose and the Van Slyke[26] method for urine ketones. Liver glycogen was determined by the Blatherwick[5] procedure and the adrenal ascorbic acid by the Roe and Kuether[22] method.

Histological.—Pancreas and adrenal tissues were placed in Zenker's fixative. Sections of pancreas were stained by Heidenhain's azan method or hematoxylin and eosin; hematoxylin and eosin were used for sections of the adrenals.

RESULTS

There was a slight drop in the blood glucose in some of the pigs for two or three days after injections were begun. If the

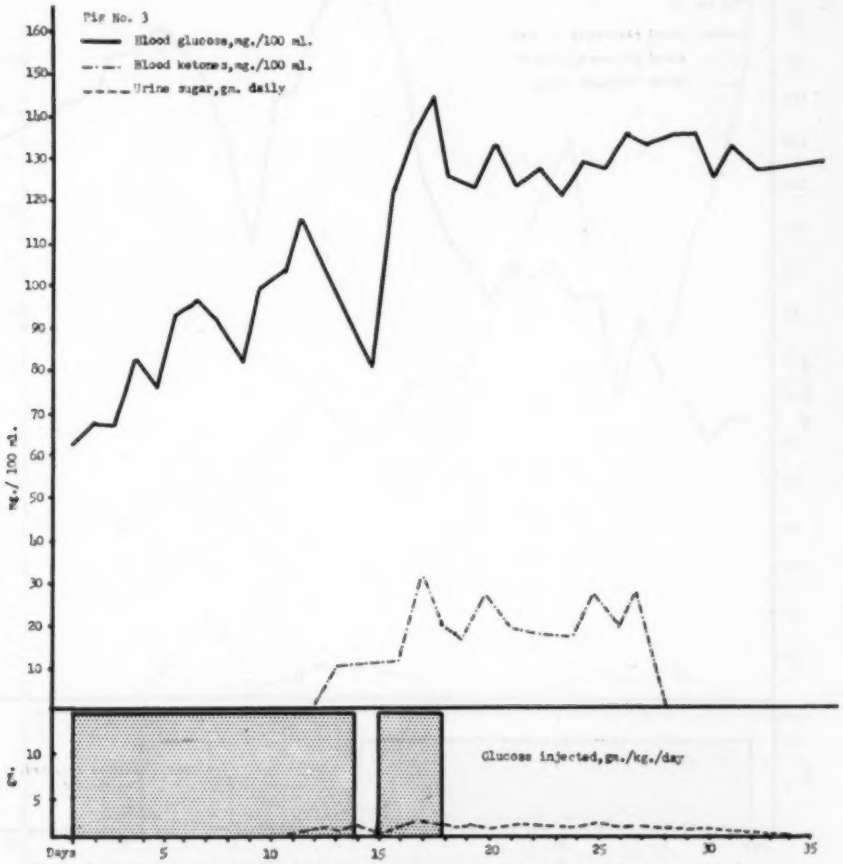

Fig. 1—The production of persistent hyperglycemia. Glycosuria was mild and persistent. The glucose tolerance curve indicated probable impairment of ability to utilize carbohydrate. The islets of Langerhans are shown in figure 6.

blood glucose was approximately double the preinjection value or above, the pig was considered to have hyperglycemia. This is an arbitrary figure but is an index of the meaning of the term as used in this discussion. Six pigs developed hyperglycemia which persisted after glucose injections were discontinued. Four of these pigs were subjected to intravenous glucose tolerance tests after a 24-hour fast, and the resulting blood glucose curves showed marked deviation from those obtained with normal pigs subjected to similar tests. Four pigs that developed pronounced hyperglycemia suddenly became weak and ataxic, then lapsed into coma from no detectable cause other than the effect of the glucose injections. Two of the 4 pigs died; the others were destroyed.

The remaining 10 pigs developed hyperglycemia of varying intensity during the course of the glucose injections, but the blood glucose returned to near the initial concentration soon after the injections were discontinued.

One striking observation was the mild glycosuria and ketosis. It is probable that the pigs which failed to develop persistent hyperglycemia may have done so had the injections been continued for a longer pe-

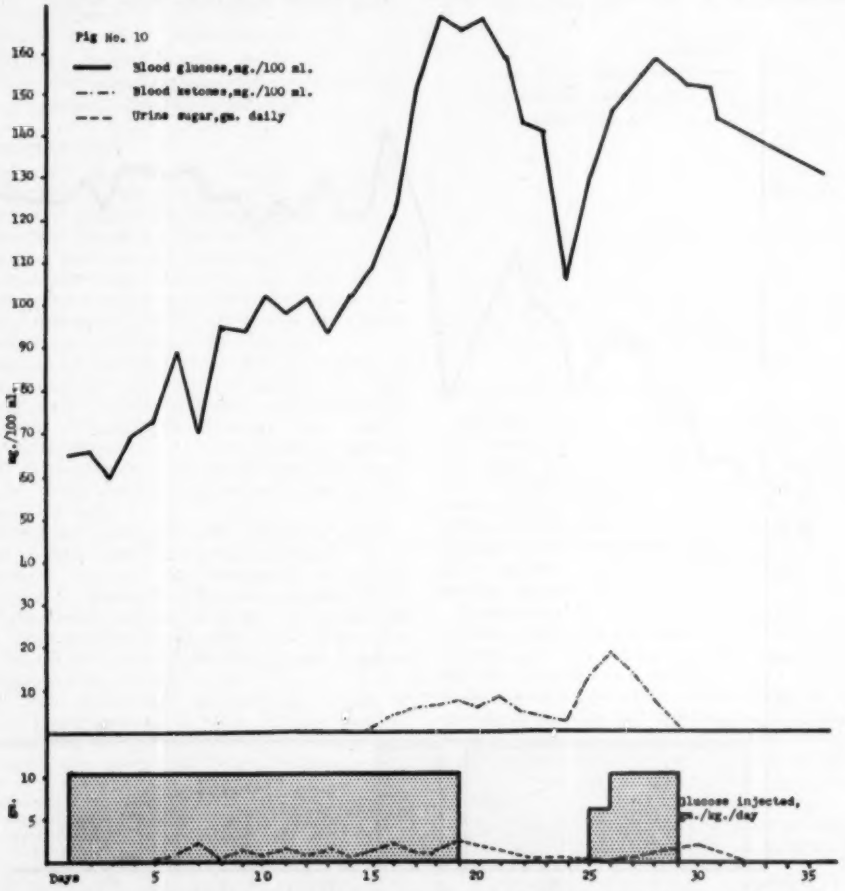

Fig. 2—The production of hyperglycemia by the administration of glucose. Note the rapid drop in blood glucose when injections were stopped and the more persistent hyperglycemia after additional injections were given.

riod. Two pigs developed rather high blood glucose concentrations of transitory character. It was not possible to predict the exact time when it was feasible to discontinue the glucose injections. Injections were discontinued in some pigs because of failure to produce marked hyperglycemia. In others, marked hyperglycemia was produced and injections were discontinued in an effort to prevent death of the animals. At autopsy, 1 pig showed evidence of staphylococcic infection. This pig did not develop hyperglycemia.

One pig gained weight during the injection period. All others lost weight even though glycosuria was mild and the pigs ate well. However, the weight loss was never greater than 8 per cent of the original body weight. Since some of the feed was spilled, it was not possible to determine accurately the exact food intake.

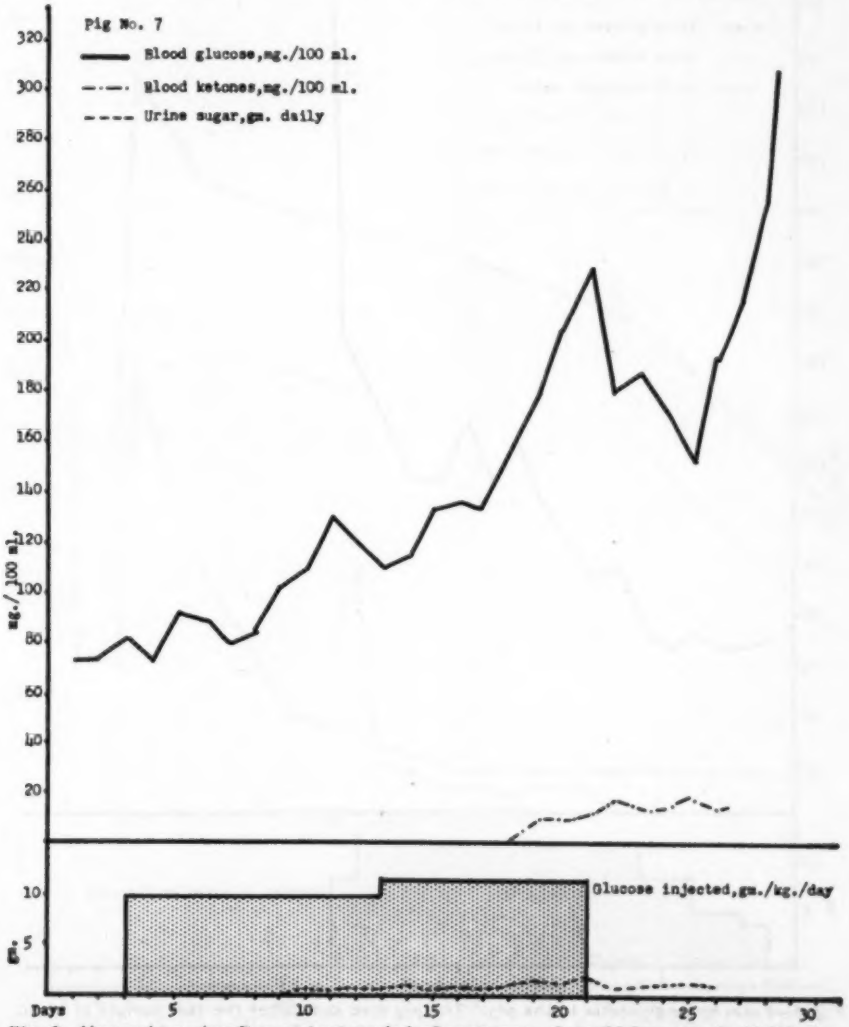

Fig. 3—Hyperglycemia after a short period of recovery. A rapid increase in blood glucose after injections were discontinued suggesting a delayed exhaustion of the islets of Langerhans.

Only 1 pig had higher blood glucose values after glucose injections were discontinued than during the injection period which lasted for seventeen consecutive days. Seven days after discontinuing injections, the pig was in a stupor and was destroyed. The blood glucose was 305.4 mg./100 ml. The pancreatic islets showed no distinct changes.

The apparent irregularity of distribution of islets of Langerhans in the pancreas of the pig made it difficult to make an accurate appraisal of the extent of injury to the islets.

One pig was observed for forty-six days after glucose injections were discontinued. The morning prefeeding blood glucose was 117.4 mg./100 ml. on the forty-sixth day

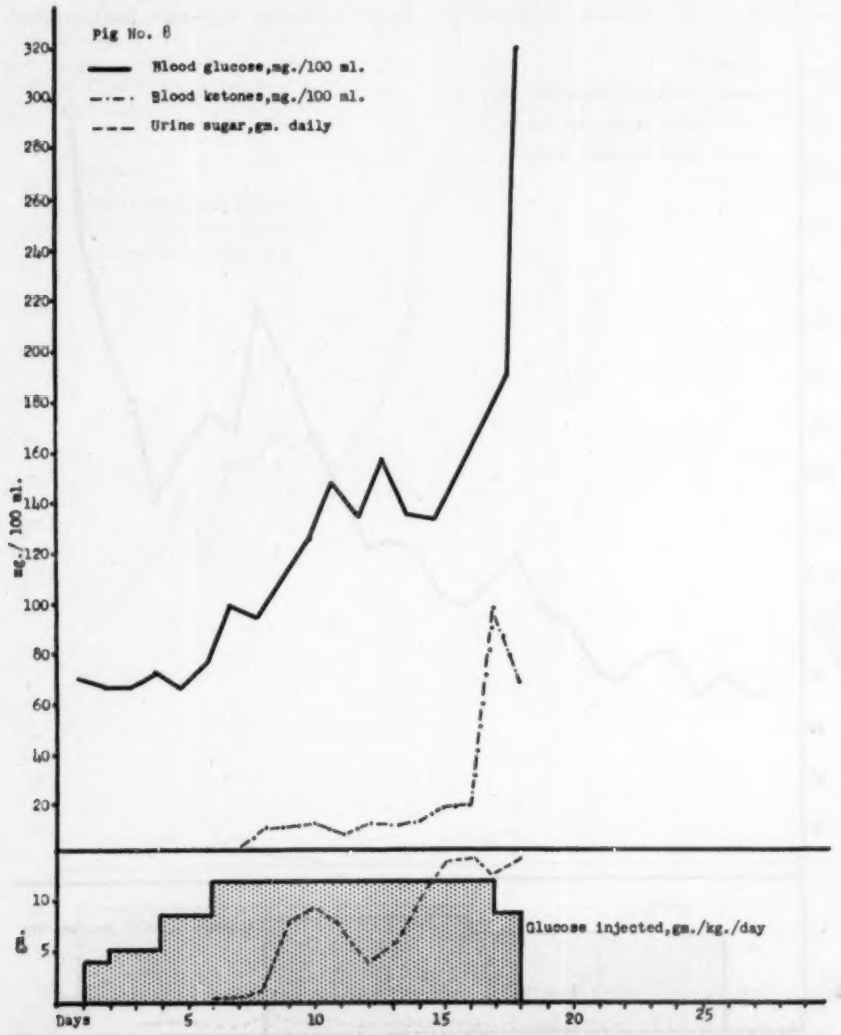

Fig. 4—Fatal hyperglycemia in the pig. The pig died soon after the last sample of blood was collected. No gross lesions were found at autopsy. Examination of the pancreatic islets revealed little if any change.

compared to 70.4 mg. at the start of the experiment. Hyperglyceemia persisted throughout this interval and glycosuria persisted throughout most of this interval. However, the morning prefeeding blood glucose did not exceed the normal renal threshold. Glycosuria indicated that the renal threshold had probably been exceeded during the period of active digestion and absorption. The pig ate well but did not gain weight. Microscopic examination of the pancreas revealed rather small islet cells with few granules, but there was no evidence of hydropic degeneration.

Data obtained from some of the pigs are graphically illustrated in the following figures. Figures 1 and 2 are typical of the 6 pigs that developed hyperglycemia which persisted after injections were discontinued. Figures 3 and 4 are representative of the pigs that became weak or comatose and died or were destroyed. Figure 1 illustrates

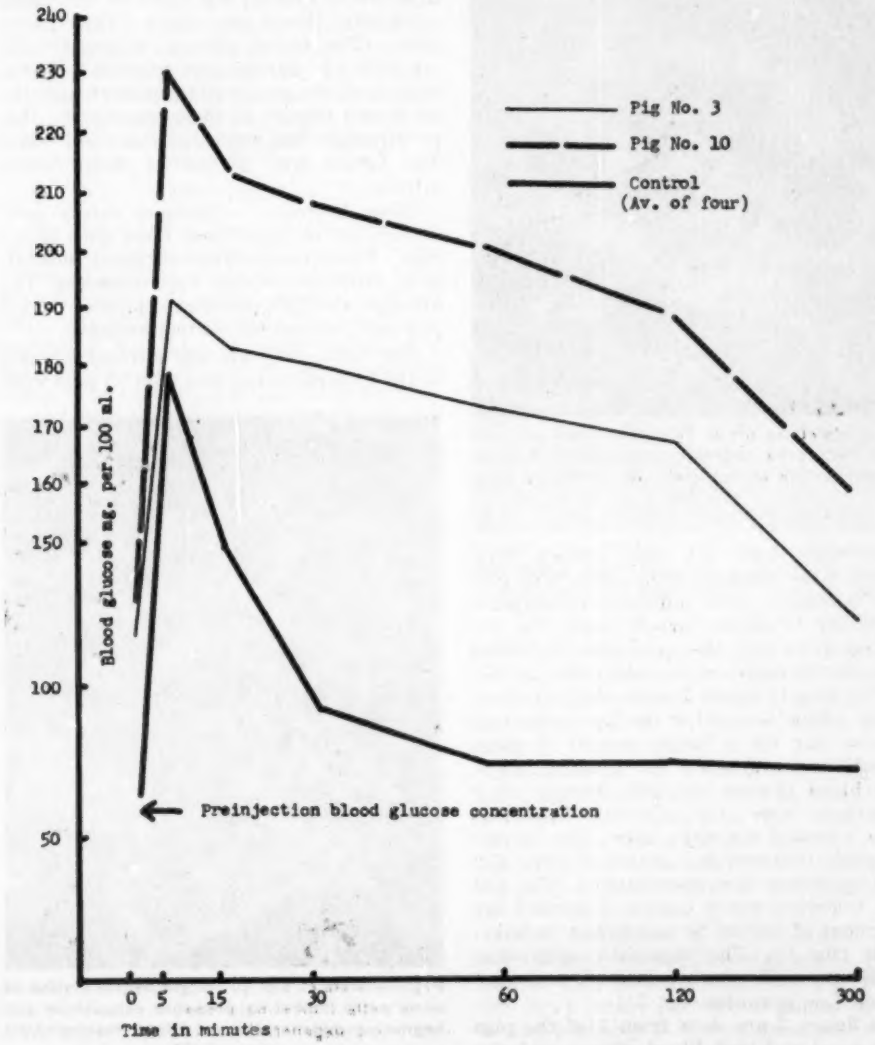

Fig. 5—Intravenous glucose tolerance test on pigs fasted twenty-four hours.

uniform hyperglycemia after glucose injections were discontinued. Glycosuria was mild but persistent while the ketosis was more pronounced than that observed in most of the pigs. Although glucose injections were made for only sixteen days, rather large amounts were given. The glucose tolerance curve indicated impairment of ability to utilize carbohydrate (fig. 5). Examination of the pancreas indicated some degeneration of the islet cells (fig. 6).

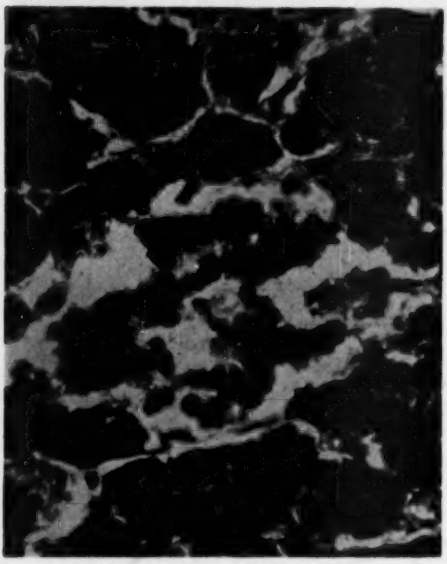

Fig. 6—Islets of pig 3. The clear areas and cell have undergone hydropic degeneration indicating exhaustion of the islets. Heidenhain's azan. x 400.

The data in figure 2 were obtained from a pig which received a smaller amount of glucose but for a longer period of time. Injections were made for nineteen days. The blood glucose dropped sharply after injections were discontinued. Injections were resumed six days later. The hyperglycemia returned and persisted after glucose injections were discontinued. The glucose tolerance curve indicated marked impairment of ability to metabolize carbohydrate (fig. 5). The pancreatic islets were small, the cells were scattered and contained few granules (fig. 7).

In figure 3 are data from 1 of the pigs that developed high blood glucose and was in a stupor six days after injections were discontinued. Even though the blood glucose was above the renal threshold, the amount of sugar excreted was relatively low. As will be noted in figure 8, some of the pancreatic islet cells appear slightly degranulated.

The data illustrated in figure 4 are from the pig which had the highest blood glucose of all the pigs used in this study. The pig was observed to be weak when the morning injection of glucose was given on the eighteenth day. Blood was collected three hours later. The blood glucose increased 127 mg./100 ml. during this interval. Examination of the pancreatic islets revealed little if any change in these structures. Hyperglycemia had persisted for eight days. The ketosis and glycosuria were rather intense.

Liver Glycogen.—Glycogen values were determined on liver tissue from some of the pigs. These tissues were obtained immediately after the animals were destroyed. The average was 0.68 per cent compared to 2.2 per cent for similar control animals.

Adrenals.—The average adrenal weights in the hyperglycemic and control pigs were

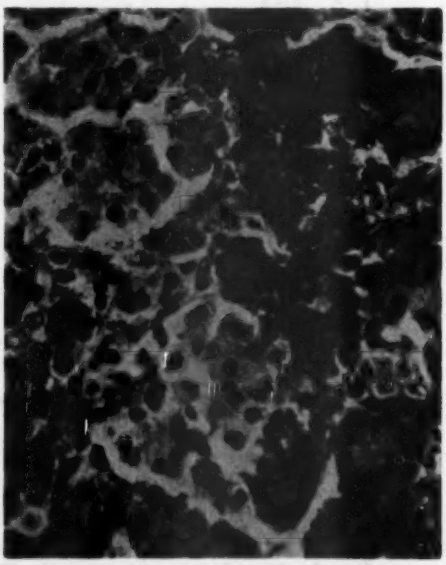

Fig. 7—Islets of pig 10. Slight degranulation of some cells indicating probable exhaustion and beginning degeneration of islets. Heidenhain's azan. x 400.

approximately 0.25 Gm./kg. of body weight. The average ascorbic acid content of the adrenal tissue was 69 mg./100 Gm. for the hyperglycemic pigs compared to 92 mg./100 Gm. for the controls. Microscopic examination of sections of the adrenals did not reveal any cellular changes.

Blood Cholesterol.—Cholesterol determinations were made on blood from some of the hyperglycemic pigs. The average value was 135 mg./100 ml. compared to the normal of 88 mg./100 ml.

One pig in which hyperglycemia had existed for nine days became weak and was given 20 units of insulin for two consecutive days. The blood glucose was lowered and life was probably prolonged by the insulin, but the case terminated fatally.

Discussion

The results show that considerable variation exists among pigs with respect to the effects of prolonged parenteral administration of glucose. It seems apparent from the data obtained in this study that the intraperitoneal injection of glucose stimulated the secretion of insulin by the pancreatic islets. The evidence for this is the decrease in the blood glucose concentration found during the first few days of each experiment. Most of the pigs showed mild inappetence during the early part of each experiment, but it is doubtful if this factor was the only cause of the reduced blood glucose concentration. Anderson and Long [2] found that the concentration of glucose in the blood circulating through the pancreas influences the secretion of insulin by that organ. Most authorities agree that hyperglycemia exhausts the insulin content of the pancreas and that a continued overstimulation may lead to degranulation and degeneration of the islet cells.

Wilder [28] refers to the hyperglycemia and glycosuria that occurs when fasted or semifasted animals or men are fed carbohydrate as "hunger" diabetes. This condition also seems to exist in fasted pigs as demonstrated by Hanawalt and Sampson.[14] Best, Haist, and Ridout [5] found that a diet low in carbohydrate reduces the amount of insulin in the pancreas. Since the majority of swine rations contain liberal amounts of carbohydrate, one would expect the pig to have a good insulin reserve. However, the hyperglycemia and glycosuria observed in pigs by Carlson and Drennen [9] and Eveleth [11] following glucose feeding may indicate that the pig has a relatively low insulin reserve.

Lack of well-defined changes in the islet cells of some of the glucose injected pigs may not be a true indication of the functional activity of the cells. The diabetic rat and rabbit show little change in the pancreatic islets. Bell [3] made an extensive

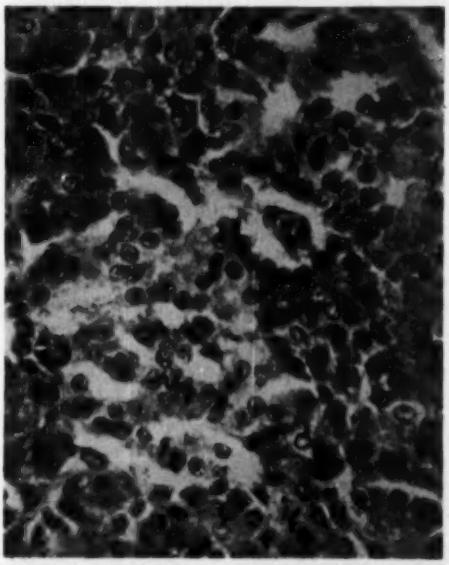

Fig. 8—Islets of pig 7. The degree of degeneration shown here does not parallel the rise in blood sugar. Hematoxylin eosin. x 400.

study of the pancreas of human beings who died from diabetes. He was unable to detect any abnormality in the appearance of the islet cells in 37 per cent of those studied.

Examination of sections made of the pancreas from 12 pigs used in this study revealed some changes in the islet cells. One of those that exhibited impaired carbohydrate tolerance when subjected to intravenous glucose tolerance tests had practically normal-appearing islets.

Guest [13] observed that alloxanized pigs did not have marked hyperglycemia when fasted. This indicates that the pig probably has a rather low rate of gluconeogenesis and probably is the explanation for the mild hyperglycemia in depancreatized

pigs. It also suggests that the pig is not dependent on insulin to the same degree as some other animals, particularly the dog and cat.

The pig is a rapidly maturing animal and its pituitary contains a high concentration of growth hormone. The growth hormone when injected into some animals causes diabetes. The pig may have a tolerance to the diabetogenic effect of the growth hormone and this may explain the rather low order of pituitary-adrenal-pancreas relationship.

The difference in the amount of cholesterol in the blood of the hyperglycemic pigs and the amount in the blood of the controls compares favorably with those reported by Lukens [12] in a pancreatectomized pig. This is also in accord with the report by Rubin [23] in which he stated that there was an increase in all blood lipids, particularly cholesterol, in the blood from human beings affected with diabetes. The reduced amount of liver glycogen in the hyperglycemic pigs also is similar to the observations made on diabetics by Mirsky *et al.*[20]

In response to a number of stimuli, there is an increased secretion of adrenotropic hormone and, hence, of adrenal cortical hormone. The measurement of this response by a fall in adrenal ascorbic acid was described by Sayers *et al.*[24] The difference in the ascorbic acid content of the adrenals taken from the experimental pigs, as compared with the ascorbic acid content of those obtained from the controls in this study, was not sufficient to indicate excessive adrenal stimulation as measured by this method.

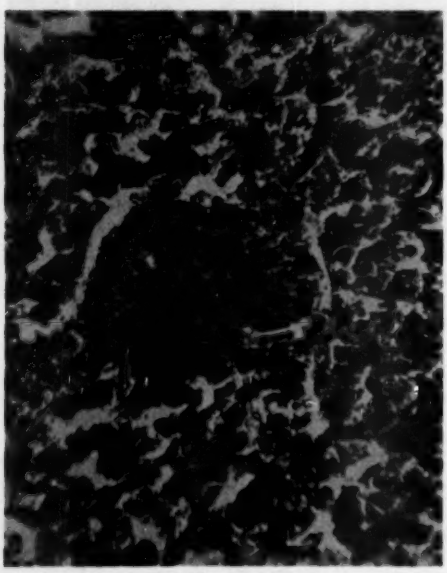

Fig. 9—Pancreatic islet of normal pig. Heidenhain's azan. x 400.

SUMMARY

Twenty apparently healthy pigs were given daily intraperitoneal injections of glucose. The injection periods ranged from twelve to thirty-four days.

Hyperglycemia persisted in 6 pigs after glucose injections were discontinued. Four other pigs became weak and comatose and died or were destroyed. The remaining 10 pigs developed mild hyperglycemia during the course of the injections, but the hyperglycemia did not persist after the injections were discontinued.

It is not known whether the hyperglycemia was permanent or temporary in those pigs that did have persistent hyperglycemia, but experimental observations in other species indicate that if hyperglycemia persists for seven or more days it is usually of a permanent nature.

Four pigs which showed persistent hyperglycemia in the postinjection period were subjected to intravenous glucose tolerance tests. The results of these tests indicated some impairment of carbohydrate tolerance.

Glycosuria, ketonemia, and ketonuria were relatively mild in most of the pigs.

Because of the apparent irregularity in the distribution of islets of Langerhans in the pancreas of the pig, it was found difficult to make accurate appraisal of the extent of injury to these structures. In some islets, there appeared to be definite variation from the normal. There was some evidence of degranulation and degeneration of the cells. Lack of evidence of more hydropic degeneration may be due partly to the relatively short duration of hyperglycemia or the anatomical appearance of the cells may not be indicative of their functional capacity.

Although the pituitary-adrenal system of the pig probably reacts to the stimulus of injected glucose, appraisal of the results obtained in this study suggest that this reaction is of low degree in this animal.

References

[1] Allen, F. M.: Experimental Studies on Diabetes. J. Metabol. Res., *1*, (1922): 5-41.

[2] Anderson, E., and Long, J. A.: Effect of Hyperglycemia on Insulin Secretion as Determined with Isolated Rat Pancreas in Perfusion Apparatus. Endocrinology, *40*, (1947a): 92-97.

[3] Bell, E. T.: The Pathological Anatomy of Diabetes Mellitus. Illinois M. J., *92*, (1947): 215-218.

[4] Benedict, S. R.: The Detection and Estimation of Glucose in Urine. J. Am. M. A., *57*, (1911): 1193-1195.

[5] Best, C. H., Haist, R. E., and Ridout, J. H.: The Influence of Diet on Insulin Content of Rat Pancreas. J. Physiol., *97*, (1939): 107-114.

[6] Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., Larson, H. W., and Sawyer, S. D.: The Determination of Tissue Carbohydrates. J. Biol. Chem., *111*, (1935): 537-547.

[7] Bloor, W. R.: The Determination of Cholesterol in Blood. J. Biol. Chem., *24*, (1916): 227-231.

[8] Carle, B. N., and Dewhirst, W. H., Jr.: A Method for Bleeding Swine. J.A.V.M.A., *101*, (1942): 495-496.

[9] Carlson, A. J., and Drennan, F. M.: A Note on the Sugar Tolerance in the Pig. J. Biol. Chem., *13*, (1912): 465-468.

[10] Dohan, F. C., and Lukens, F. D. W.: Experimental Diabetes Produced by the Administration of Glucose. Endocrinology, *42*, (1948): 244-262.

[11] Eveleth, D. F.: The Blood Chemistry of Swine. I. Blood Changes Following the Ingestion of Glucose. J. Biol. Chem., *104*, (1933): 559-563.

[12] Gould, L. K., and Carlson, A. J.: Further Studies on the Relation of the Pancreas to the Serum and Lymph Diastases. Am. J. Physiol., *29*, (1911): 165-181.

[13] Guest, G. A., Cincinnati, Ohio: Personal communication. 1950.

[14] Hanawalt, V. M., and Sampson, J.: Studies on Baby Pig Mortality. IV. Chemistry of Blood During Fasting and Refeeding of Weanling Pigs. Am. J. Vet. Res., *8*, (1947): 73-81.

[15] Hanawalt, V. M., Link, R. P., and Sampson, J.: Intravenous Carbohydrate Tolerance Tests in Swine. Proc. Soc. Exptl. Biol. and Med., *65*, (1947): 41-44.

[16] Homans, J.: Degeneration of the Island of Langerhans Associated with Experimental Diabetes in the Cat. J. Med. Res., *30*, (1914): 49-68.

[17] Jacobs, H. R., and Colwell, A. R.: Lesions in the Pancreas and in the Anterior Hypophysis with Fatal Acidosis Following Prolonged Intravenous Administration of Glucose (in Dogs). Am. J. Physiol., *116*, (1936): 194-200.

[18] Lukens, F. D. W.: Pancreatectomy in the Pig. Am. J. Physiol., *118*, (1937): 231-327.

[19] Minkowski, O.: Diabetes Mellitus Nach Pankreasexstirpation. Arch. f. exp. Path. u. Pharmakol., *31*, (1893): 93-97.

[20] Mirsky, J. A., Korenberg, M., Nelson, N., and Nelson, W. E.: The Hepatic Glycogen Reserves in Diabetes Mellitus. Endocrinology, *28*, (1943): 358-367.

[21] Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., *153*, (1944): 375-380.

[22] Roe, J. H., and Kuether, C. A.: The Determination of Ascorbic Acid in Whole Blood and Urine Through the 2,4 Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid. J. Biol. Chem., *147*, (1943): 401-409.

[23] Rubin, S. H.: The Plasma and Red Blood Cells Lipids in Persistent (Diabetic) Lipemia and in Transient (Alimentary) Lipemia. J. Biol. Chem., *31*, (1939): 691-695.

[24] Sayers, C., Sayers, M. A., Liang, T. Y., and Long, C. N. H.: Effect of Pituitary Adrenotrophic Hormone on Cholesterol and Ascorbic Acid Content of Adrenals of Rat and Guinea Pig. Endocrinology, *38*, (1946): 1-9.

[25] Somogyi, M.: Determination of Blood Sugar. J. Biol. Chem., *160*, (1945): 61-68.

[26] Van Slyke, D. D.: The Determination of B-hydroxybutyric Acid, Acetoacetic Acid, and Acetone in Urine. J. Biol. Chem., *35*, (1917): 455-458.

[27] Van Slyke, D. D., and Fitz, R.: Studies of Acidosis. VIII. The Determination of B-hydroxybutyric Acid, Acetoacetic Acid, and Acetone in Blood. J. Biol. Chem., *32*, (1917): 495-497.

[28] Wilder, R. N.: Reflections on the Causation of Diabetes Mellitus. J. Am. M. A., *114*, (1950): 1234-1239.

[29] Woerner, C. A.: Effect of Continuous Intravenous Injection of Dextrose in Increasing Amounts on Blood Sugar Level, Pancreatic Islands and Liver of Guinea Pigs. Anat. Rec., *75*, (1939): 91-105.

Studies on Aureomycin Therapy of Brucellosis in Swine

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THERAPEUTIC agents for combatting brucellosis have long interested physicians and veterinarians. Arsenicals, mercurochrome, methylene blue, specific vaccines and bacterins, artificially induced fever, nonspecific foreign protein therapy, serum therapy, sulfonamides, and the antibiotics have been tried with varying reports of success and failure. Most of the reputable therapeutic agents tried have appeared to be successful in some cases, but none has always been successful. The nature of the disease and the difficulties of diagnosis make the evaluation of treatment difficult.

Efforts have been made in this study to evaluate the use of aureomycin in swine. Because of the similarity of the disease in swine and man, the findings may indicate the therapeutic activity in man.

REVIEW OF LITERATURE

Holm and Moore [1] reported that aureomycin and aureomycin-sulfadiazine reduced the size of spleens in artificially infected guinea pigs. The combination was more toxic than aureomycin alone.

Braude and Spink [2] reported that aureomycin protected mice from a lethal dose of Brucella. Werner and Knight [3] found that streptomycin and aureomycin had antibrucellar action in mice.

Spink *et al.*,[4] Spink and Yaw,[5] Braude *et al.*,[6] and Ralston and Payne [7] reported some apparent success with aureomycin in treating *Brucella melitensis* and *Brucella abortus* infections in man.

Green and Stein [8] reported failure with aureomycin treatment of three people infected with *Brucella suis*.

Larson and Gilman [9] and Bryan and Boley [10] failed to eliminate *Br. abortus* from cattle by means of aureomycin.

Cameron [11] believed that the aureomycin in an aureomycin-fermentation residue affected a bacteriological cure in 13 of 14 swine, while *Br. suis* was isolated from 8 of 10 nontreated controls.

EXPERIMENTAL PROCEDURE

Three trials were made. Forty-five swine of various ages were used. Aureomycin hydrochloride was used in two of the trials. The third study was made with "aurofac," a fermentation residue of the commercial production of aureomycin.

Bacteriological and serological examinations were made as described by Hutchings *et al.*[12] At autopsy, bacteriological examinations were made of liver, spleen, kidneys, heart, lung, bladder, and popliteal, prefemoral, superficial inguinal, internal iliac, mesenteric, gastrohepatic, mandibular, cervical, and suprapharyngeal lymph nodes. The testes, epididymides, prostate, Cowper's glands, and seminal vesicles were examined in the males, and the ovaries and uterus in the females.

Blood serum levels of aureomycin were determined by the method of Dornbush and Pelcak.[13]

Trial 1.—Ten swine, naturally infected as evidenced by agglutination reactions, weighing between 175 and 300 lb., were used. Five of the animals were treated with aureomycin hydrochloride, using an initial dose of 5 mg. per pound of body weight and a sustaining dose of 2½ mg. per pound of body weight twice daily. The antibiotic was given intraperitoneally. Treatment was continued for ten days. The remaining swine served as controls.

Agglutination tests and hemacultures were made at the start of treatment and prior to slaughter. Blood levels of aureomycin were determined at intervals. At autopsy, four days after treatment stopped, bacteriological examinations of the tissues were made for *Br. suis*.

Trial 2.—Ten pigs, weighing from 30 to 90 lb., were exposed to *Br. suis* by intravenous injection. Treatment was started eighteen days following exposure after infection had been established by agglutination tests and hemacultures. Aureomycin hydrochloride was given intraperitoneally to 5 of the pigs, using an initial dose of 10 mg. per pound of body weight and a sustaining dose of 5 mg. per pound of body weight. The remaining 5 pigs served as controls. The treatment was continued for twelve days, when the animals were killed. Agglutination tests and hemacultures were made twice weekly following exposure and bacteriological examinations were made at autopsy. Blood levels of aureomycin were determined at intervals.

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Journal paper 623, Purdue University Agricultural Experiment Station, Lafayette.

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The authors acknowledge the assistance of Dr. C. R. Smith, School of Veterinary Medicine, Ohio State University, Columbus.

Trial 3.—The results of the first two trials indicated that aureomycin might have some therapeutic effect. It was thought that intermittent therapy might enhance the value of the agent. Also, it was desired to learn the course of the infection following therapy if the animals were kept for a time after treatment stopped.

Twenty-five shoats, negative to agglutination tests and weighing from 31 to 53 lb., were exposed to *Br. suis* by spraying their mouths with approximately 301,500,000 organisms in mucin. The animals were divided into five groups. Group 1 was to be treated once and killed immediately for bacteriological study. Group 2 was to serve as control for group 1. Group 3 was to be treated intermittently three times, and killed. Group 4 was to be treated once and killed eight weeks later. Group 5 served as a control for groups 3 and 4.

Treatment of groups 1, 3, and 4 was started twenty-three days after exposure. It was estimated that each shoat would eat about 3 lb. of feed per day. The three groups were started on self-feeders containing aurofac in feed. Three pounds of feed contained an equivalent of 1 Gm. of aureomycin hydrochloride. All three groups were kept on this treatment for twelve days. One week after treatment was stopped, groups 1 and 2 were killed for bacteriological examination.

After a lapse of two weeks, the same treatment was repeated for twelve days for two successive times in group 3. Groups 3, 4, and 5 were killed ten days after the end of the last treatment and examined bacteriologically.

Agglutination tests and hemacultures were made at weekly intervals following exposure. Blood levels of aureomycin were determined at irregular intervals.

RESULTS

Table 1 shows the results of trial 1. There was no change in the agglutination response of the treated or control pigs during the course of the study. *Brucella suis* was not isolated from the blood of any of the animals at any time during the study. At autopsy, *Br. suis* was recovered from only 1 of the treated pigs and 2 of the controls.

The blood serum levels of aureomycin ranged between 0.25 and 32.0 μg. per milliliter, most of the values falling between 1.5 and 2.0 μg. per milliliter. The results of this trial were thought to be inconclusive, probably due to the stage of the infection at the time of treatment. The indications are that the active infection had subsided naturally prior to the treatment.

Table 2 shows the results of trial 2. No changes were noticed in agglutinin production following treatment. *Brucella suis*

TABLE 1—Results of Experimental Aureomycin Therapy of Brucellosis in Swine, Trial 1.

Pig (No.)	Postexposure maximum titer* and No. positive hemacultures	Post-treatment maximum titer and No. positive hemacultures	No. of tissues positive for *Brucella suis* at autopsy
	Treated		
73	400 0	400 0	1
74	200 0	200 0	0
78	200 0	400 0	0
554	200 0	400 0	0
66	100 0	100 0	0
	Controls		
81	200 0	200 0	0
594	200 0	200 0	1
2932	100 0	50 0	0
571	400 0	200 0	1
68	400 0	200 0	0

* Serum agglutination tests were conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400.

TABLE 2—Results of Experimental Aureomycin Therapy of Brucellosis in Swine, Trial 2.

Pig (No.)	Postexposure maximum titer* and No. positive hemacultures	Post-treatment maximum titer and No. positive hemacultures	No. of tissues positive for *Brucella suis* at autopsy
	Treated		
211	400 1	400 0	6
346	400 1	400 0	4
379	400 2	400 0	4
307	400 2	400 0	7
365	400 3	400 0	9
	Controls		
344	400 2	400 2	24
262	400 2	400 2	15
257	400 3	400 2	12
315	400 2	400 3	23
340	400 2	400 3	15

* Serum agglutination tests were conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400.

was recovered from the blood of each animal following exposure. *Brucella suis* was isolated from the blood of all of the controls but from none of the treated pigs after treatment started. At autopsy, *Br. suis* was recovered from the tissues of all 10 animals, but fewer isolations were made from the treated pigs than from the untreated ones.

The blood serum levels of aureomycin ranged between 0.0 and 8.0 μg. per milliliter with most of the values between 1.5 and 2.0 μg. per milliliter.

Tables 3 and 4 show the results of trial 3. The administration of aurofac had no effect in changing the agglutination titers. Although immediate cessation of bacteremia following initiation of therapy was not noted in this trial, the number of isolations following therapy was lowered. Again it was noted that fewer isolations of *Br. suis* were made at autopsy from the treated swine. The intermittent treatments seemed to enhance the ability of aureomycin to eliminate the *Br. suis* from the body tissues, since only one isolation was made from 1 animal (table 4).

TABLE 3—Results of Experimental Aureomycin Therapy of Brucellosis in Swine, Trial 3.

Pig (No.)	Postexposure maximum titer* and No. positive hemacultures	Post-treatment maximum titer and No. positive hemacultures	No. of tissues positive for *Brucella suis* at autopsy
Group 1: Treated once—killed immediately following end of treatment for bacteriological study of tissues			
1023	50 1	50 0	1
1025	50 1	50 0	0
1029	100 3	100 2	3
1035	0 1	0 0	1
1045	50 2	50 0	2
Group 2: Untreated controls for group 1			
1043	100 2	50 3	16
1053	200 3	200 4	14
1105	25 3	50 5	13
1127	50 2	50 3	17
3669	200 2	200 3	15

* Serum agglutination tests were conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400.

The blood levels of aureomycin during therapy ranged from 0.25 to 12.8 μg. per milliliter, with the majority between 1.5 and 2.0 μg. per milliliter.

DISCUSSION

Aureomycin did not alter agglutinin production in treated animals as compared with the controls.

Following the administration of aureomycin, as aureomycin hydrochloride or aurofac, fewer isolations of *Br. suis* were obtained from treated as compared with untreated animals. *Brucella suis* was isolated at least once from the blood of 6

TABLE 4—Results of Experimental Aureomycin Therapy of Brucellosis in Swine, Trial 3.

Pig (No.)	Postexposure maximum titer* and No. positive hemacultures	Post-treatment maximum titer and No. positive hemacultures	No. of tissues positive for *Brucella suis* at autopsy
Group 3: Three intermittent treatments—killed following third treatment			
1027	50 1	100 1	1
1034	100 1	200 1	0
1041	200 3	100 0	0
1048	200 3	200 2	0
3667	0 0	0 0	0
Group 4: Treated once—killed eight weeks following end of treatment			
1031	100 1	100 2	1
1038	100 1	100 2	1
1049	50 2	100 0	0
1051	50 2	100 0	1
1102	50 1	50 0	2
Group 5: Untreated controls for groups 3 and 4			
3643	50 3	100 5	3
3659	25 1	25 0	1
3661	25 1	400 3	2
3677	50 2	200 5	1
3685	100 3	400 5	2

* Serum agglutination tests were conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400.

(24.0 %) of 25 treated swine as compared with 14 (70 %) of 20 untreated controls.

At autopsy, *Br. suis* was isolated from at least one tissue of 15 (60 %) of 25 treated swine and from at least one tissue of 17 (85 %) of 20 controls. The tables show that in many cases *Br. suis* was found in fewer tissues of the individually treated animals than of the individual controls. Of 5 animals treated intermittently, *Br. suis* was recovered from only one tissue of 1 animal as compared with recovery from 5 controls in that group (table 4).

The above results indicate that aureomycin did not cure all *Br. suis* infected swine, as indicated by bacteriological and serological findings. However, aureomycin or aurofac may have had some beneficial effect. Further experiments and field trials are necessary to confirm or deny this assumption.

SUMMARY

1) The administration of aureomycin did not alter the agglutinin production by *Brucella suis* infected swine as compared with the antibody response of controls.

2) *Brucella suis* was isolated from the blood at least once following the initiation of aureomycin treatment of infected swine from 6 (24 %) of 25 treated swine as compared to recoveries from 14 (70 %) of 20 of the untreated controls.

3) *Brucella suis* was isolated at autopsy from at least one tissue from 15 (60 %) of 25 aureomycin-treated infected swine and from at least one tissue of 17 (85 %) of 20 controls.

4) In the opinion of the authors, further experiments and field trials are necessary to confirm or deny the effectiveness of aureomycin in the treatment of swine brucellosis.

References

[1] Holm, L. W., and Moore, W. C.: Studies on the Efficacy of Aureomycin and Aureomycin-Sulfadiazine in Experimental Brucellosis in Guinea Pigs. Am. J. Vet. Res., *11*, (1950): 211-213.

[2] Braude, A. I., and Spink, W. W.: The Action of Aureomycin and Other Chemotherapeutic Agents in Experimental Brucellosis. J. Immunol., *65*, (1950): 185-199.

[3] Werner, C. A., and Knight, V.: The Suppressive Effect of Antimicrobial Drugs on *Br. melitensis* Infection in Mice. J. Immunol., *65*, (1950): 509.

[4] Spink, W. W., Braude, A. I., Castaneda, M. R., and Coytia, R. S.: Aureomycin Therapy in Human Brucellosis Due to *Brucella melitensis*. J. Am. M. A., *138*, (1948): 1145-1148.

[5] Spink, W. W., and Yaw, E. M.: Aureomycin: Present Status in the Treatment of Human Infections. J. Am. M. A., *141*, (1949): 964-966.

[6] Braude, A. I., Hall, W. H., and Spink, W. W.: Aureomycin Therapy in Human Brucellosis Due to *Brucella abortus*. J. Am. M. A., *141*, (1949): 831-835.

[7] Ralston, R. J., and Payne, E. H.: Treatment of Chronic Brucellosis with Chloramphenicol and Aureomycin. J. Am. M. A., *142*, (1950): 159-161.

[8] Green, T. W., and Stein, L.: Failures with Aureomycin in the Treatment of Infection with *Brucella suis*, J. Am. M. A., *144*, (1950): 380-383.

[9] Larson, P. H., and Gilman, H. L.: Aureomycin as a Treatment of Acute Brucellosis of Cattle. Cornell Vet., *40*, (1950): 259-272.

[10] Bryan, H. S., and Boley, L. E.: Aureomycin in the Treatment of Bovine Brucellosis. Vet. Med., *46*, (1951): 158-159.

[11] Cameron, H. S.: The Bactericidal Action *in vivo* of Aureomycin in an Aureomycin Fermentation Residue Against *Brucella suis*. Cornell Vet., *41*, (1951): 110-114.

[12] Hutchings, L. M., Bunnell, D. E., Donham, C. R., and Bay, W. W.: The Viability of *Brucella suis* in Swine Carcasses. Proceedings Book, AVMA. (1950): 184-189.

[13] Dornbush, A. C., and Pelcak, E. J.: The Determination of Aureomycin in Serum and Other Body Fluids. Ann. New York Acad. Sci., *51*, (1948): 218.

Studies on the Vaccination of Swine Against Brucellosis

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BECAUSE of the importance attached to vaccination as a means of controlling diseases in general and because of the hope that an effective vaccine may be found to produce a reliable immunity to brucellosis, these vaccination studies were undertaken.

LITERATURE CITED

Various workers have reported that a previous infection seems to decrease the susceptibility of swine to subsequent infection. McNutt [1] in discussing this problem says: "Hogs are naturally resistant to both *abortus* and *melitensis* and often show considerable resistance to *suis* strains . . . it is thought that recovery from one attack renders the animals less susceptible to subsequent infection."

Hadley and Beach [2] considering the same problem say: "Infectious abortion in swine appears to be a self-limiting disease in that a naturally acquired infection usually is followed by immunity."

Holm, Ardrey, and Beeson [3] found vaccination of young pigs with *Brucella abortus* strain 19 gave satisfactory results. These results disagreed with the findings of Manthei [4] in controlled experiments with swine and with Kernkamp and Roepke [5] with field cases of brucellosis in swine.

Hutchings *et al.* [6] found that hogs previously exposed to *Brucella suis* by either natural or experimental means were not as responsive to a second exposure but that the resistance was not sufficient to prevent reinfection.

The antigenic properties of endoantigens of Brucella cells have been reported upon by various workers. [7-10]

Live [11] and Live and Danks [12] have described the immunizing properties of ether-killed *Br. abortus* plus an adjuvant in guinea pigs and in cattle. In both studies, the adjuvant-vaccine was promising. In the study with cattle, the vaccine compared favorably with strain 19.

Larson [13] reported on the immunization of guinea pigs with a soluble antigen, extracted with ether, incorporated into Freund's adjuvant with the addition of heat-killed human tubercle bacilli. He was able to protect from 80 to 100 per cent of guinea pigs in the study against multiple infective doses of virulent *Br. abortus*.

Huddleson [14] reported on the successful immunization of guinea pigs with organisms of the mucoid phases of *Br. suis* and *melitensis*.

The use of Brucella M vaccine in cattle in field trials was reported by Clark and Phelps [15] and Killham *et al.* [16] Edgington and King [17] have reported on the progress of controlled experiments with cattle using this agent. None of the authors at the time of these reports made conclusive statements as to the effectiveness of the vaccine.

The mucoid vaccine study was conducted at the request of the Conference of Research Workers in Swine Brucellosis held at Purdue University in May, 1949.

ETHER AND PHENOL-SOLUBLE EXTRACTS OF BRUCELLA CELLS AS IMMUNOGENIC AGENTS AGAINST BRUCELLOSIS IN SWINE

Experimental Procedure.—Thirty swine, about three months of age, were used for this study. The animals were negative to agglutination tests for brucellosis. Five animals were vaccinated with 1 cc. of the ether extract; 5 with 2 cc. of the ether extract; 5 with 1 cc. of the phenol extract; 5 with 2 cc. of the phenol extract. The vaccines were administered subcutaneously in the axillary space. The remaining 10 animals served as unvaccinated controls. The vaccinated and control animals were kept together on pasture. Agglutination tests were conducted on all swine at weekly intervals following vaccination. Hemacultures were made three weeks following vaccination.

Three months following vaccination, each animal of all groups was exposed to approximately 2,000,000,000 *Br. suis* organisms in mucin by spraying the oral cavity.

Agglutination tests and hemacultures were made at intervals up to the time of slaughter.

All of the animals were destroyed for postmortem studies between two and three months after exposure. Bacteriological examinations of the tissues were made for *Br. suis*.

The serological and cultural examinations of the blood, and the bacteriological examination of tissues were made as described by Hutchings *et al.* [18]

Tissues examined bacteriologically at autopsy were: popliteal, prefemoral, superficial inguinal, internal iliac, mesenteric, gastrohepatic, bronchial,

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The phenol and ether soluble extracts of Brucella cells were prepared and furnished by Dr. N. B. Carle and Dr. C. L. Larson, National Institute of Health Laboratories, Bethesda, Md.

The Brucella M vaccine was furnished by Dr. I. F. Huddleson, Central Brucella Laboratory, Michigan State College, East Lansing.

TABLE 1—Results of Exposure of 10 Swine to Brucella Suis Following Vaccination with Ether Extract of Brucella Suis Cells

No. and sex*	Maximum post-vaccinal titer**	Titer at exposure	Postexposure agglutination titer and blood culture results †					Autopsy results		
			Time in days after exposure					Days after exposure	Aggl. titer blood culture	No. tissues positive *Br. suis*
			6	16	21	28	42			
					1 cc. vaccine					
229F	+++PT	-----	-----	++T-- Pos.	PT--- Pos.	++PT-	PPT--	70	+++P-	7
267F	++T--	-----	-----	----- Pos.	-----	+PT-- Pos.	PPTT- Pos.	83	++PT-	6
247F	+++PT	-----	-----	T----	-----	++PT- Pos.	++PT-	85	+++PT	2
316M	++++P	-----	-----	+PT-- Pos.	PPT-- Pos.	++++P Pos.	+++T-	54	+++PT	30
387M	+++PT	-----	-----	T---- Pos.	PT---	+++PT	+++TT Pos.	56	+++PT	22
					2 cc. vaccine					
233M	+++PT	T----	TT---	T----	-----	PPT-- Pos.	+PT-- Pos.	54	PTT-- Pos.	31
318M	++++P	-----	T----	++T--	++PT-	++PT- Pos.	++PT- Pos.	56	+PT--	20
237M	+++PT	T----	T----	PT---	PP---	++PT-	+PTT- Pos.	85	++++P	28
359F	++P--	PT---	+PT--	++PT-	PT---	PT---	PPT--	70	++PT-	0
325F	+PT--	-----	-----	PPT--	T----	PT---	PT--- Pos.	83	PT---	0

* F—female; M—male.

** Serum agglutination tests conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400; +—complete agglutination; P—partial agglutination; T—trace of agglutination; —— no agglutination.

† Pos.—recovery of *Br. suis* from blood stream.

TABLE 2—Results of Exposure of 10 Swine to Brucella Suis Following Vaccination with Phenol Extract of Brucella Suis Cells

No. and sex*	Maximum post-vaccinal titer**	Titer at exposure	Postexposure agglutination titer and blood culture results †					Autopsy results		
			Time in days after exposure					Days after exposure	Aggl. titer blood culture	No. tissues positive *Br. suis*
			6	16	21	28	42			
					1 cc. vaccine					
312F	+++++	-----	-----	T----	PT--- Pos.	+++PT Pos.	+++PT	70	++++P	8
337M	+++PT	T----	-----	----- Pos.	T---- Pos.	PTT-- Pos.	PT--- Pos.	54	+++PT Pos.	17
329M	+++PT	++PT-	++T--	++P--	+PT--	++PT-	PT---	56	PT--- Pos.	2
363M	+++++	-----	T----	++---	+PT--	++PT-	+PT--	85	T---- Pos.	2
333F	+++PT	T----	T----	TT---	TT---	PPT-- Pos.	++P-- Pos.	83	++PT-	3
					2 cc. vaccine					
347F	+++PT	T----	-----	TT---	T----	PT---	T----	70	PT---	4
255M	++++P	T----	-----	P---- Pos.	++PT-	+PT--	++PT-	56	++P--	1
249F	+++PT	T----	-----	+PT--	PP---	++PT-	++PT-	83	++++-	1
253M	++++P	T----	T----	+PT--	PT---	++++P Pos.	++++P	56	+++PT	3
245F	++++P	TT---	T----	++PT-	+++PT	+++PT Pos.	++PT- Pos.	83	++PT-	2

* F—female; M—male.

** Serum agglutination tests conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400; +—complete agglutination; P—partial agglutination; T—trace of agglutination; —— no agglutination.

† Pos.—recovery of *Br. suis* from blood stream

mandibular, cervical, and suprapharyngeal lymph nodes; liver, spleen, kidney, heart, lungs; in females, ovary and uterus; in males, testis, epididymis, prostate, seminal vesicles, and Cowper's gland. In the case of paired organs or lymph nodes both were cultured.

Procedure for preparation of the cellular extract vaccines was described by Larson.[18]

RESULTS

The results of the use of phenol and ether extracts of Brucella cells for immunogenic agents are shown in tables 1 and 2. Table 3 contains the results of exposure of the controls.

All the vaccinated animals developed significant agglutination reactions which receded to essentially negative within the three-month period between vaccination and exposure. No Brucella was isolated from any of the animals before experimental exposure.

Following exposure, the agglutinin titers in the animals in all groups rose to a diagnostic level, though on the whole they were not as high as the reactions observed in the vaccinated animals before exposure.

Only 1 animal (359, table 1) failed to yield *Br. suis* at any time following exposure. This animal was in the group vaccinated with 2 cc. of the ether extract.

The average number of isolations from the tissues at autopsy made from the swine in the group vaccinated with the phenol extract was lower than the number isolated from either the ether extract group or the control group. It will be noted from the tables, also, that *Br. suis* was recovered less frequently from the blood streams of those animals receiving the 2-cc. amounts of the cellular extracts. These observations may or may not be evidence of increased resistance due to the vaccine. From the data presented, however, we must conclude that the materials used as vaccines did not protect the swine from subsequent infection with *Br. suis*.

BRUCELLA M VACCINE AS AN IMMUNOGENIC AGENT AGAINST BRUCELLOSIS IN SWINE

Experimental Procedure.—Sixty shoats with negative agglutination tests were divided into two groups. One group of 40 animals was injected subcutaneously with 1 cc. of Brucella M vaccine (serial lot 60 c). The remaining 20 nonvaccinated swine served as controls. The two groups were kept together in a barn and adjoining pasture which had not been used by Brucella-infected animals for at least eight years. Agglutination tests and hemacultures were conducted on all the swine at irregular intervals for about five months, after which time the tests were conducted weekly until all remaining swine were destroyed.

The female swine were bred to Brucella-free boars, starting at about five months after vaccination. At farrowing time, the fetal membranes,

TABLE 3—Results of Exposure of 10 Unvaccinated Swine to Brucella Suis

No. and sex*	Titer at exposure**	Postexposure agglutination titer and blood culture results †					Autopsy results		
		Time in days after exposure					Days after exposure	Aggl. titer blood culture	No. tissues positive *Br. suis*
		6	16	21	28	42			
310F	T————	T———— Pos.	++TT— Pos.	T————	TT——— Pos.	PT———	70	PPT——	0
259M	————	————	TT———	T———— Pos.	PT——— Pos.	+++P— Pos.	56	++++P Pos.	24
329F	————	T————	PT——— Pos.	PT———	+PT—— Pos.	PTT——	83	+PT——	5
205M	T————	————	++P———	+PT—— Pos.	++PT—	PT———	56	PT———	6
308M	————	————	T————	TT——— Pos.	+++P— Pos.	TT———	85	++PT—	18
231F	T————	————	++PT— Pos.	PPT—— Pos.	+++PT Pos.	++++P	70	++++P	2
352F	————	————	————	———— Pos.	————	T————	83	+PT——	17
301M	————	————	————	T———— Pos.	+PT——	PT——— Pos.	85	+++PT	17
474M	————	————	PTT——	++P——— Pos.	++PT—	PT———	85	+PT——	1
263M	————	Not bled	TT——— Pos.	+PT—— Pos.	++++P Pos.	++PT—	85	+++PT	14

* F—female; M—male.

** Serum agglutination tests conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400; +—complete agglutination; P—partial agglutination; T—trace of agglutination; —no agglutination.

† Pos.—recovery of *Br. suis* from blood stream.

tissues of dead and weak pigs, and vaginal swabs were examined bacteriologically for *Br. suis*.

Eight months following vaccination, all of the remaining swine were exposed by spraying the oral cavity with 1 cc. of a saline suspension containing about 97,500,000 *Br. suis* organisms mixed with 4 cc. of gastric mucin. The strain of *Br. suis* used for exposure was isolated from the blood of animal 659, 1 of the vaccinated group, following vaccination.

The remaining animals were destroyed for bacteriological examination of the tissues from between three and four months after exposure. The serological and bacteriological techniques used were the same as described by Hutchings *et al.*[18]

RESULTS

This study was complicated by an outbreak of swine dysentery which was first observed thirteen days after vaccination. Eleven vaccinated and 2 control swine died of dysentery. Since there had been some indication that streptomycin and aureomycin were effective against dysentery,[19] the 20 control pigs were treated with these antibiotics in order to prevent as much death loss as possible. The vaccinated group was not treated for fear treatment might effect the immunogenic action of the vaccine. The lack of treatment is believed to be the reason for the higher death loss in the vaccinated group than in the controls.

Three additional animals either died or were killed due to extraneous causes prior to exposure to *Br. suis*. The autopsy reports on the animals which died prior to exposure will be found in table 4.

TABLE 4—Autopsy Reports on Swine Which Died or Were Killed Prior to Exposure to Brucella Suis

No. and sex*	Experimental status	Diagnosis from autopsy
707F	Control	Died, dysentery
633F	Vaccinated	Died, dysentery
757F	Vaccinated	Died, dysentery
767B	Vaccinated	Died, dysentery
701F	Vaccinated	Died, dysentery
741F	Vaccinated	Died, dysentery
683F	Vaccinated	Died, dysentery
613F	Vaccinated	Died, dysentery
715B	Vaccinated	Died, dysentery
709B	Vaccinated	Died, dysentery
635B	Vaccinated	Died, dysentery
713F	Vaccinated	Died, dysentery
653F	Control	Died, dysentery
621F	Vaccinated	Killed, severe ear infection
641F	Vaccinated	Died, gastroenteritis, suggilated area on left ventricle. Examined bacteriologically, no Brucella isolated.
725B	Vaccinated	Died, vegetative endocarditis. Examined bacteriologically, no Brucella isolated.

* F—female; B—barrow.

Following vaccination but prior to deliberate exposure, positive hemacultures for *Br. suis* were obtained from 3 of the vaccinated swine and from 3 of the controls, as shown in table 5. It should be noted that the first isolations of *Br. suis* were made from animals in the vaccinated group. The first isolation of *Br. suis* from 1 of those vaccinated, before deliberate exposure, was made about three months prior to any other isolation of *Br. suis*. It should be emphasized that at no time during the preceding eight years had there been Brucella-infected animals in the pens or adjoining pasture in which these animals were kept.

Except in the cases of the animals which appeared to be actively infected, the agglutination responses following vaccination were transient, the maximum titers are shown in the third columns of tables 5 and 6. They appeared to be the same in both the vaccinated and control groups.

After exposure to *Br. suis*, positive hemacultures were obtained from 24 of 26 vaccinated swine and from 15 of 18 control swine. Two of the 3 animals in the control group from which *Br. suis* was not isolated following exposure were animals which had previously been actively infected as evidenced by positive hemacultures. Likewise, the 2 animals in the vaccinated group from which no *Br. suis* was isolated had shown positive hemacultures prior to experimental exposure.

At the time of slaughter or death, bacteriological examination of the tissues of these two groups of swine yielded *Br. suis* from 13 of 26 vaccinated swine and from 8 of 18 control swine, as shown in tables 5 and 6. *Brucella suis* was not recovered from 2 of the 3 vaccinated swine and 2 of the 3 control swine which had developed a bacteremia prior to deliberate exposure.

No abortions were noted but exposure occurred late in pregnancy. *Brucella suis* was not recovered from any of the fetal membranes, tissues of dead and weak pigs, or from the vaginal swabs examined at farrowing.

A spondylitis was observed in 1 animal of each group (645 and 623). *Brucella suis* was isolated from the spinal lesion in each case. *Brucella suis* was isolated from an abscess in the neck region of 1 of the

control swine (783). Temporary or intermittent lameness was noted in both groups after experimental exposure to *Br. suis*.

DISCUSSION

The administration of phenol and ether extracts of Brucella cells as vaccines produced high agglutinin titers in the vaccinated animals, while no such reaction was observed in the controls. The rise in titer observed in the Brucella M vaccine study, after vaccination of the principals, was of the same degree in both the vaccinated and control groups. Although the titers in the latter study were not as high as in the cell extract study, it must be noted that these animals were not bled as frequently immediately following vaccination due to the complicating dysentery infection. The establishment of agglutinin titers in the cases of the agents used in these trials did not indicate the production of immunity. Nor did the absence of abortions in the swine vaccinated with the mucoid vaccine indicate lack of infection as indicated by the ability to isolate the organisms from the blood and from tissues at autopsy.

It will be recalled from the vaccination trial with the mucoid vaccine that *Br. suis* was not isolated after exposure from 5 of the animals which showed evidence of infection after vaccination but prior to deliberate exposure. These observations lend credence to the theory that individual animals may show considerable resistance to reinfection.

In the opinion of the authors, there are two possible explanations for the establishment of infection prior to experimental exposure in the animals of the Brucella M vaccine experiment: (1) contaminated environment due to the fact that many previous studies with *Br. suis* had been conducted on neighboring premises; and (2) the introduction of a viable organism as an immunizing agent. Although the animals had been housed in the same area for six weeks prior to vaccination, the first evidence of infection was observed three and one-half weeks after vaccination.

TABLE 5—Results of Exposure of 26 Swine to Brucella Suis Following Vaccination with Brucella M Vaccine

No. and sex*	Maximum prevaccination titer**	Maximum postvaccination titer	No. postvaccination positive blood cultures	Titer at time of exposure	Maximum postexposure titer	No. postexposure positive blood cultures	Autopsy time days after exposure	No. tissue isolation of *Br. suis*
659B	------	+++PT	5	+P---	+++P-	0	51	0
658F	------	PPT--	2	------	PPT--	0	98	0
687B	------	------	0	------	PT---	6	91	0
850B	------	T-----	0	------	++PT-	2	98	1
627B	------	------	0	------	+++PT	4	98	1
728F	------	T-----	0	------	+PT--	3	35	2
655B	------	------	0	------	++PT-	5	88	0
719F	------	PT----	0	T-----	++PT-	6	88	0
721B	------	PT----	0	T-----	+++PT	2	51	0
3883F	------	PPT---	0	------	++PT-	6	102	2
794B	------	T-----	0	------	+PPT-	4	88	4
669B	------	TT----	0	------	+PT--	6	102	0
771F	------	------	0	------	++PT-	5	105	0
842F	------	------	0	T-----	+++++	6	102	2
665F	------	T-----	0	------	+++PT	8	105	1
761F	------	PT----	0	PT----	++PT-	4	53	13
697F	------	T-----	0	------	+PT--	5	51	2
661F	------	T-----	0	------	++PT-	5	109	4
705B	------	T-----	1	------	+PT--	3	109	0
615F	------	PTT---	0	------	++PT-	5	109	0
464B	------	T-----	0	------	++PT-	7	109	2
712B	------	+PP---	0	+PT---	++PT-	5	91	0
629B	------	TT----	0	------	++PT-	8	111	2
645B	------	PPT---	0	T-----	++PT-	6	91	3
733F	------	++PT-	0	PT----	++PT-	2	111	0
675B	------	T-----	0	------	++PT-	5	111	0

* B—barrow; F—female.

** Serum agglutination tests conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400. +—complete agglutination; P—partial agglutination; T—trace of agglutination; ——no agglutination.

The possibility of a reversion of the avirulent mucoid variant *Br. suis* to a virulent type of organism in its natural host is suggested by the fact that some of the animals became actively infected prior to deliberate exposure as evidenced by positive hemacultures. Furthermore, the first evidence of infection after vaccination was in the vaccinated group, from whence it appeared to spread to the control group. It must be emphasized, however, that although the animals were kept on premises which had not been used within the previous eight years to house Brucella-infected animals, considerable other work with *Br. suis* infected animals had been conducted on closely surrounding areas.

Some of the recovered strains were sent to Dr. Huddleson for observation. In regard to these strains Dr. Huddleson reported: "Examination of these cultures on tryptose agar containing basic fuchsin reveals that they are in no way related to the smooth culture of *Brucella suis* from which the mucoid growth phases were derived. Growth of all of your cultures were completely inhibited by basic fuchsin in tryptose agar.

"The smooth culture from which the mucoid phases were derived and all of its dissociated growth phases grow as well on tryptose agar containing basic fuchsin as on tryptose agar containing thionin. . . . If the mucoid phases used in the vaccine ever revert to the smooth phase, I am sure that they will grow as well on tryptose basic fuchsin agar as on the original culture."

SUMMARY

1) Under the conditions of this experiment, the phenol and ether extracts of *Brucella suis* cells which were used to vaccinate 20 swine did not protect the swine against subsequent exposure to *Br. suis*.

2) Such phenol and ether extracts were agglutinogenic as indicated by agglutination tests conducted following vaccination.

3) Under the conditions of this experiment Brucella M vaccine did not protect 23 of 26 vaccinated swine against subsequent exposure to *Br. suis*.

4) Three swine vaccinated with Brucella M vaccine and 3 control animals kept with the vaccinated swine became infected following vaccination but prior to deliberate exposure.

5) Five of 6 swine which became infected prior to deliberate exposure showed evidence of resistance to subsequent exposure to *Br. suis*.

TABLE 6—Results of Exposure of 18 Unvaccinated Swine to Brucella Suis

No. and sex*	Maximum titer before vaccination of principals**	Maximum titer after vaccination of principals	No. postvaccination positive blood cultures	Titer at time of exposure	Maximum postexposure titer	No. postexposure positive blood cultures	Autopsy time days after exposure	No. tissue isolations of *Br. suis*
745B	------	------	1	------	+++PP	9	91	0
783B	------	T-----	0	------	++++T	6	98	2
775B	------	TT----	0	------	+++PT	3	91	0
787F	------	+PT---	0	+PT---	+PT---	0	98	0
759B	------	+++PT	1	++T---	++PT-	0	51	0
619F	------	PT----	0	------	+++PT	8	102	6
763B	------	TT----	0	------	+++PT	2	102	0
735B	------	------	0	------	++++P	5	54	5
695B	------	++PT-	2	T-----	TT----	0	88	0
791F	------	T-----	0	------	+PT---	2	105	1
601F	------	T-----	0	------	++++P	7	51	6
605F	------	PT----	0	------	++PT-	4	105	0
623F	------	TT----	0	------	++PT-	3	38	25
679F	------	++PT-	0	------	+++PT	3	59	10
663F	------	PT----	0	T-----	+++PT	5	105	2
789F	------	PT----	0	------	++++P	3	109	0
691B	------	T-----	0	------	+++PT	3	110	0
699F	------	------	0	------	+PT---	1	27	0

* B—barrow; F—female.

** Serum agglutination tests conducted at dilutions of 1:25, 1:50, 1:100, 1:200, and 1:400. +—complete agglutination; P—partial agglutination; T—trace of agglutination; ——no agglutination.

References

[1] McNutt, S. H.: Brucella Infection in Swine. Proc. 42nd Ann. Meet., U. S. Livestock San. A., (1938): 90-97.

[2] Hadley, F. B., and Beach, B. A.: An Experimental Study of Infectious Abortion in Swine. Univ. of Wisconsin Agric. Exper. Sta. Res. Bull., *55*, 1922.

[3] Holm, G. C., Ardrey, B. B., and Beeson, W. M.: A Vaccination Program for the Control of Swine Brucellosis. Proc. 49th Ann. Meet., U. S. Livestock San. A., (1945): 191-196.

[4] Manthei, C. A.: Research on Swine Brucellosis by the Bureau of Animal Industry (1941-1947). Am. J. Vet. Res., *9*, (1948): 40-43.

[5] Kernkamp, H. C. H., and Roepke, M. H.: Vaccination of pigs with *Brucella abortus* Vaccine Strain 19. J.A.V.M.A., *113*, (1948): 564-567.

[6] Hutchings, L. M., Delez, A. L., and Donham, C. R.: Studies on Brucellosis of Swine. II. Exposure and Re-exposure Experiments with *Brucella suis*. Am. J. Vet. Res., *7*, (1946): 11-20.

[7] Miles, A., and Pirie, N. W.: The Properties of Antigenic Preparations from *Brucella melitensis*. Brit. J. Exptl. Path., *20*, (1939): 83-98.

[8] Paterson, J. S., Pirie, N. W., and Stableforth, A. W.: Protective Antigens isolated from *Brucella abortus*. Brit. J. Exptl. Path., *28*, (1947): 223-236.

[9] Pennell, R. B., and Huddleson, I. F.: Quantitative Studies of Brucella Precipitin Systems. I. Precipitation of Homologous Antisera by Brucella Endoantigens. J. Exptl. Med., *68*, (1938a): 73-81.

[10] Pennell, R. B., and Huddleson, I. F.: II. Precipitation of Heterologous Antisera by Brucella Endoantigens. J. Exptl. Med., *68*, (1938b): 83-93.

[11] Live, I.: Effect of Adjuvants upon the Immunizing Quality of Ether-Killed *Brucella abortus*. Am. J. Vet. Res., *10*, (1949): 347-350.

[12] Live, I., and Danks, A. G.: The Use of Ether-Killed *Brucella abortus* in Saline-in-Oil Emulsion in a Herd of Cattle with Brucellosis. Am. J. Vet. Res., *12*, (1951): 175-182.

[13] Larson, C. L.: Immunization of Guinea Pigs with a Soluble Antigen Obtained from *Brucella abortus*. J. Immunol., *63*, (1949): 471-477.

[14] Huddleson, I. F.: The Immunization of Guinea Pigs with Mucoid Phases of Brucella. Am. J. Vet. Res., *8*, (1947): 374-379.

[15] Clark, C. F., and Phelps, L.: Observations on the Use of Brucella (M) Vaccine. Proc. 53rd Ann. Meet. U. S. Livestock San. A., 1949: 29-35.

[16] Killham, B. J., Reed, G. W., and Clark, C. F.: Field Experiences with Brucella (M) Vaccine. Proc. 53rd Ann. Meet., U. S. Livestock San. A., 1949: 25-28.

[17] Edgington, B. H., and King, N. B.: Progress Report on the Use of Brucella (M) Vaccine Under Controlled Experimental Conditions. Proc. 53rd Ann. Meet., U. S. Livestock San. A., 1949: 36-42.

[18] Hutchings, L. M., Bunnell, D. E., Donham, C. R., and Bay, W. W.: The Viability of *Brucella suis* in Swine Carcasses. Proceedings Book, AVMA, (1950): 184-189.

Renal Glucose Threshold in the Pig

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RATIONS FED to swine in the Cornbelt of the United States usually contain much corn, a cereal that is approximately 70 per cent starch.[7]

Carlson and Drennan [1] reported that the normal pig has a low tolerance for carbohydrate given by mouth. Hanawalt, Link, and Sampson [6] made observations on the tolerance of swine for carbohydrate, and concluded that the growing pig has a tolerance curve similar to that of normal human subjects. Critical data on the renal glucose threshold in swine are not available. Data have been presented on the renal glucose threshold in cattle by Bell and Jones [4] and for the horse by Link.[6]

PROCEDURE

The study reported herein on the renal glucose threshold in swine was made on 15 healthy pigs ranging in weight from 3 kg. to 52.7 kg. (6.6 to 116 lb.). Trials were made on successive days with 4 pigs to determine whether there was daily variation in kidney function and whether the time interval after feeding altered the renal threshold. All pigs were provided adequate fluids before the trials to insure active urine secretion.

Seven of the smaller pigs used in this study were anesthetized by intravenous injection of nembutal. The ureters were then cannulated through a laparotomy incision to allow collection of urine as soon as secretion began.

Eight female pigs were not anesthetized but were restrained in a wooden trough in a prone position. A catheter was introduced into the bladder. The bladder was drained and specimens of urine collected at definite intervals during the experiment. It was found that an interval of approximately four minutes elapsed between the secretion of urine and the time it reached the bladder. Therefore, tests on the urine collected four or more minutes after the injection of glucose were considered reliable indicators whether the threshold had been reached.

Blood samples were drawn from the anterior vena cava by the technique of Carle and Dewhirst.[5] To produce hyperglycemia, a 50 per cent solution of glucose was injected into the anterior vena cava. Blood samples were collected at one, two, four, and twelve minutes after completion of each glucose injection. A separate syringe was used for each procedure. The initial trial with each pig was made by injecting a quantity of glucose calculated to be sufficient to raise the blood sugar to about 150 mg./100 ml. If the renal threshold was not exceeded, additional glucose was injected to raise the blood sugar concentration. If the urine test indicated that a large amount of reducing sugar was present, the trial was terminated and the data discarded. Only those trials in which the urine sugar did not exceed 30 mg./100 ml. were considered valid and the data recorded.

The blood glucose values accepted as the renal threshold were those concentrations observed when only small amounts of reducing sugar appeared in the urine. The true threshold would probably be slightly less than these figures. Blood sugar determinations were made by the Somogyi [8] method. The Benedict [2] method was used to detect reducing sugar in the urine.

RESULTS

In some of the trials, the blood glucose concentration was raised much higher than the renal threshold; in others, the hyperglycemia was insufficient to cause glycosuria. The results of these trials are not included in figures 1 and 2. Only the trials in which the renal glucose threshold was slightly exceeded, as indicated by the results of the quantitative Benedict test on the urine for reducing sugar, are reported.

There appeared to be some relationship between the size and age of the pig and the renal threshold. Young pigs have a higher blood glucose concentration than older pigs. It appears logical to assume that they may also have a higher renal glucose threshold. Results obtained in the trials reported herein substantiate this assumption.

Seven pigs weighing from 3.0 to 14.1 kg. (average 8.7) had renal thresholds ranging from 149.7 mg./100 ml. to 169.0 mg./100 ml. with an average of 160.1 mg./100 ml. blood. Results of these trials are illustrated in figure 1. One pig that weighed 3 kg. had the highest threshold as well as the highest initial blood sugar concentration. It was not determined whether nembutal had any effect on the renal threshold in these pigs. Reducing sugar was present in the urine collected from the ureters two minutes after injection of glucose.

Eight pigs weighing from 21.3 to 52.7 kg. (average 36.4) had renal thresholds

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from 142.4 mg./100 ml. to 158.0 mg./100 ml. with an average of 147.2 mg./100 ml. blood. Results of these trials are illustrated in figure 2. There appeared to be little variation in the threshold in those pigs weighing over 35 kg. Trials were made on 4 pigs on successive days. There was only slight variation in the results obtained. The time interval after feeding did not seem to alter the renal threshold. Successive trials on the same pig gave similar results.

Discussion

The renal glucose threshold has been determined for the horse, cow, and pig. The values are remarkably similar although there is considerable variation in the normal blood glucose concentrations among these different species. The data reported herein and that reported by Hanawalt, Link, and Sampson [5] suggest that the pig has a normal carbohydrate tolerance similar to that for human beings and that an appreciable increase in the blood glucose can occur before the renal threshold is exceeded.

The ability of the kidney to reabsorb glucose from the glomerular filtrate depends upon (1) the inherent ability of the tubules to transfer the glucose, (2) the concentration of glucose in the filtrate, and (3) the rate at which the filtrate is formed. There is evidence to indicate that the transfer of the glucose is accomplished by an enzymatic process in which there is a limiting factor imposing a maximal rate on the tubular reabsorption of glucose. The threshold may vary with certain condi-

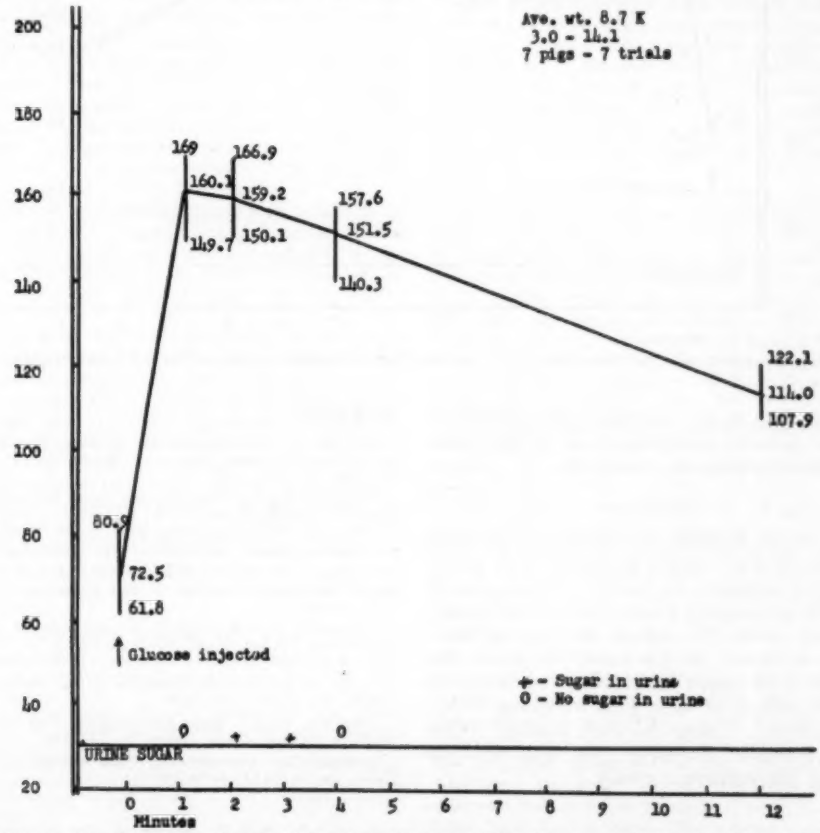

Fig. 1—Renal glucose threshold for 7 small pigs. Pigs there anesthetized with nembutal and urine collected from ureters.

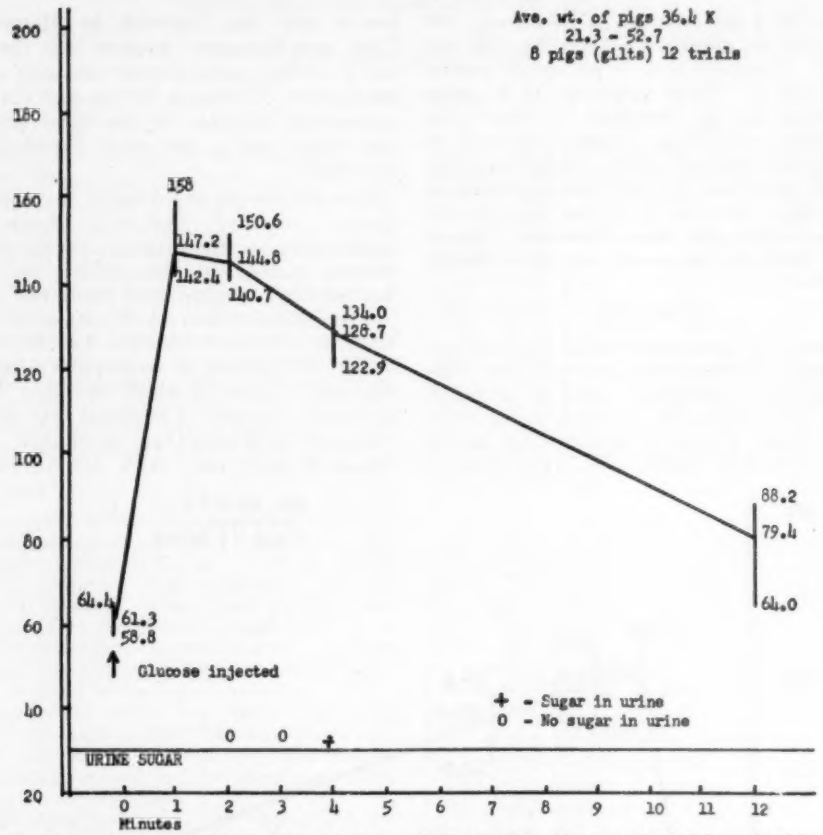

Fig. 2—Renal glucose threshold for 8 unanesthetized pigs. Urine collected from bladder.

tions, and it is, therefore, impossible to draw definite conclusions as to the exact threshold under all conditions.

SUMMARY

A report is made of a study of the renal threshold for glucose in swine. The growing pig appears to have a renal glucose threshold ranging from 142.4 to 169.0 mg./100 ml. blood. The age of the pig may have some influence on the renal threshold but since there seems to be little variation in the threshold in those pigs weighing 35 kg. and above, it may be that mature swine have a similar threshold. No data were obtained for mature animals.

References

[1] Bell, F. R., and Jones, E. R.: Glucose Tolerance in the Bovine. J. Comp. Path. and Therap., *55*, (1945): 117-124.

[2] Benedict, S. R.: The Detection and Estimation of Glucose in Urine. J. Am. M. A., *57*, (1911): 1193-1195.

[3] Carle, B. N., and Dewhirst, Wm. H., Jr.: A Method for Bleeding Swine. J.A.V.M.A., *101*, (1942): 495-496.

[4] Carlson, A. J., and Drennan, F. M.: A Note on the Sugar Tolerance in the Pig. J. Biol. Chem., *13*, (1912-13): 465-466.

[5] Hanawalt, Vera M., Link, R. P., and Sampson, Jesse: Intravenous Carbohydrate Tolerance Tests on Swine. Proc. Soc. Expti. Biol. and Med., *65*, (1947): 41-44.

[6] Link, R. P.: Glucose Tolerance in the Horse. J.A.V.M.A., *97*, (1940): 261-262.

[7] Morrison, F. B.: Feeds and Feeding. 20th ed. The Morrison Publishing Co., Ithaca, N. Y. 1942.

[8] Somogyi, M.: Determination of Blood Sugar. J. Biol. Chem., *160*, (1945): 61-68, 69-73.

The Bromsulfalein Liver Function Test and Biopsy of the Liver in the Diagnosis of Senecio Poisoning in Cattle

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No satisfactory method of diagnosis of Senecio poisoning in the living animal has been available. Animals do not live more than forty-eight hours after signs of illness appear. If a satisfactory method could be found for diagnosing this condition, these animals could be salvaged for food since the principal lesion is advanced liver cirrhosis. Methods of diagnosis used in determining liver cirrhosis in cattle are herein described.

Methods

Bromsulfalein Test for Liver Damage.—Bromsulfalein is a dye (phenoltetrabromphthalein sodium sulfonate) that, when injected intravenously, is removed from the blood by the liver and is then excreted principally through the urine. The rate at which the normal liver removes this dye from the circulating blood has been well established for the human being. Severe damage to the liver slows the removal of this dye from the blood.

This test was selected because it has been reported to indicate chronic liver damage better than most tests.[1] It is simple and is adaptable for use upon cattle. Many of the other tests are less accurate and are more difficult with intractable cattle.

As used in this work, the weight of the animal when divided by 30 gave a figure which was the number of cubic centimeters of the dye solution* to be injected. It has 50 mg. of the powdered dye in each cubic centimeter of solution. Original directions were to divide the weight of the human body by 22 to get the number of cubic centimeters of dye solution to be used. When this amount was used in normal cattle some of it still remained in the blood after thirty minutes. For that reason, the time for blood sampling was lengthened to forty-five minutes and the volume of dye solution was computed by dividing the weight of the animal by 30.

Routinely, blood samples were taken at fifteen-minute and forty-five-minute intervals after injection. The blood was allowed to clot at room temperature; the clot was loosened and the samples were then centrifuged at 2,000 r.p.m. for ten minutes. Two cubic centimeters of the serum were then withdrawn, placed in tubes 1 cc. in diameter, and 2 drops of 10 per cent sodium hydroxide were added to each tube. Upon addition of this alkali, a purple color developed in those cases where some of the dye remained in the blood serum. These tubes were immediately compared with the color standards. When severe damage to the liver was encountered, most of the dye was still in the blood serum. For example, 30 per cent of the dye still remained in the blood serum of calf 56 twenty hours after injection.

Liver Biopsy.—Since a great deal of time was necessary in running the bromsulfalein liver function test on each animal, other more practical tests were sought. A Swedish trochar (Wall's hepatic trochar[2]) was obtained and used in taking liver biopsies from animals which were on a Senecio-silage feeding experiment. The small pieces of liver taken in this way were examined microscopically.

Technique of Performing the Biopsy.—A 4-inch square area was clipped on the right side of the animal. The exact point of operation was located by counting the ribs from the last one forward. The point selected for the incision was just anterior to the eleventh rib (third from the last). The distance from the dorsal median line varied with the size of the animal but in general it was 27 or 28 cm. in mature cattle. This distance was reduced to approximately 20 cm. for the average yearling of 500 lb.

After thorough aseptic preparation of the site, an incision 2 cm. long was made with a sharp scalpel in the skin midway between the tenth and eleventh ribs.

After making the skin incision, the intercostal muscles and the pleural membrane were incised with a stabbing movement of the knife. Following puncture, a wheezing noise was noticed since, in breathing, air was drawn through this opening and forced out. The hepatic trochar, with its stylet in place, was then inserted through the incision. Rotation of the instrument aided in getting it into the pleural cavity. It was then pushed inward, slightly downward and backward until it encountered a firm mass which moved anteriorly and posteriorly with each exhalation. After feeling this firm mass, which was the liver covered by the diaphragm, the stylet was removed from the instrument and the finger placed as a stop 2½ inches away from the incision. The trochar was then thrust into the mass, the knife, which is a part of

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* Supplied only by Hynson, Westcott, and Dunning, Baltimore, Md.

the trochar, was pushed down to cut off the piece of liver inside the trochar and the instrument was removed. The stylet was then used to push out the liver sample which was placed in a small tea strainer—with an identification number—and immediately immersed in a 10 per cent formalin solution for fixation.

Each wound was wiped free of blood with dry cotton and E. Q. 335 was smeared over the entire area to prevent screw-worm infestation. No further care was given and all animals healed without incident. Ear tags identified the animals.

The biopsy samples remained in the tea strainers until they were ready to be embedded for sectioning. The tissues were then embedded in paraffin (Fischer's tissue-mat) and stained by the hemotoxylin-eosin method.

RESULTS

The bromsulfalein liver function test was first run on 8 cattle in the experiment station herd. Some of these were 300-lb. calves and some were almost 3 years old. Some showed 30 to 40 per cent dye retention after thirty minutes so the proper time to take the blood samples was considered to be at fifteen- and forty-five-minute intervals. Some showed retention at thirty minutes. This was considered correct for normal cattle.

Calf 56 showed no retention of the dye in his blood when tested Feb. 2, 1950 (table 1). He was then fed 1 per cent of his body weight of *Senecio longilobus* each day for ten days. On March 29, 1950, the test was repeated. At that time, 15 per cent of the dye was retained fifteen minutes after injection, but none remained in forty-five minutes. When retested April 5, he was showing signs of poisoning. He had a diarrhea and was very thin and weak. At that time, all the dye was retained in the blood after forty-five minutes. Autopsy, April 20, showed typical pathology of Senecio poisoning.

Another animal shown in table 1 (Rhinehart bull) was found in a prostrate condition on a ranch near the experiment station thirty-six hours previously. The animal's weight was estimated at 1,200 lb. The dye was injected and 100 per cent was retained forty-five minutes later. Decholin sodium* was then injected intravenously. This drug is a liver stimulant expected to increase the blood flow to, and the bile flow from, the liver. Thirty cubic centimeters of 20 per cent sodium salt of dehydrocholic acid solution was used. Within twenty minutes this animal was able to assume sternal recumbency. He drank 10 gal. of water and ate 2 lb. of range cubes. Twelve hours later he was again prostrate and straining; he was destroyed and an autopsy was performed. Typical gross pathology for Senecio poisoning was found. In addition, several hematomas and large bruised areas were found. These were assumed to be due to injuries inflicted by other bulls in the same pasture. After autopsy, it was concluded that this bull probably would have lived several weeks longer had he not been in a fight. Thus, the test undoubtedly indicated correctly that the liver was functioning poorly several weeks before the animal probably would have died.

Table 1 also shows the results of this liver function test on calves 170 and 168. Calf 170 was fed 22.5 lb. (9.8 % of its body weight) of green *Senecio longilobus* between May 3, 1952, and May 24, 1952, given at the rate of 1.2 lb. per day with a balling gun. When the last liver function test was made, on May 30, this animal was not very active but no other abnormalities could be seen. He was showing typical symptoms of poisoning June 7, and died June 15. Typical lesions of Senecio poisoning were found upon autopsy except that he showed more icterus than seen in most experimental cases.

At the same time that the above animal was on experiment, calf 168 was fed 10.3 lb. (9.1 % of its body weight) of green *Senecio riddellii*. No retention of the dye

TABLE 1—Bromsulfalein Tests on Animals Poisoned with Senecio Experimentally and on Field Cases

Date	Animal (No.)	5 Min. (%)	15 Min. (%)	45 Min. (%)
2–2–50	56	100	0	0
3–29–50	56	100	15	0
4–5–50	56	100	100	100
4–6–50	56	20 hours after inj. 4–5–50: 30 % retention		
10–5–50	Rhinehart bull	No test	100	100
5–2–52	168	No test	0	0
5–26–52	168	No test	Tr.	0
5–30–52	168	No test	Tr.	0
6–20–52	168	No test	0	0
7–14–52	168	No test	Tr.	0
5–2–52	170	No test	Tr.	0
5–26–52	170	No test	60	0
5–30–52	170	No test	80	50

Tr.—trace.

* Ames Co., Inc., Elkhardt, Ind.

was noted on this animal on June 20. For that reason, between June 28 and July 3, an additional 11½ lb. (5 % of its body weight) of the same plant was fed at the rate of 1 per cent of the body weight per day. This animal appeared to be normal July 14 when the last tests were run but the bromsulfalein test indicated that a mild acute hepatitis existed at that time.

Calf 147 died December 17 and calf 143 died Dec. 22, 1951. Both showed typical straining some twenty-four hours before death. Autopsy revealed typical fibrosis of the liver in both, but 143, which lived a week longer, showed more extensive fibrosis. Microscopic examination after death revealed rapidly progressing cirrhosis in the livers of both. In both animals, a positive diagnosis by liver biopsy was possible several weeks before death. Calf 168 also showed some increase in fibrosis several weeks before and a true cirrhosis on the final biopsy on July 14.

TABLE 2—Liver Biopsies on Calves Poisoned with Green Plants of Senecio Longilobus and Senecio Riddellii

Animal (No.)	Date	When taken	Microscopic pathology
143	10–22–51	3 days before fed any green Senecio.	Normal.
143	11–20	First time after scouring.	Lipidosis, necrosis of many cells and starting fibrosis; liver beginning to look porous.
143	12–8	2 weeks before death.	Marked fibrosis and some lipidosis; senecio poisoning.
143	12–22	Dead.	Autopsy—firm cirrhotic liver; 1 gal. intraperitoneal fluid.
147	10–22–51	3 days before fed any green Senecio.	Normal.
147	11–10	First time after scouring.	Fibrosis is more prominent than in 143; little lipidosis; liver architecture is still visible.
147	12–17	Dead.	Autopsy—extremely cirrhotic liver; edema of mesentery.
168	4–20–52	5 days before fed any green Senecio.	Essentially normal.
168	5–26	After being fed 9.1 % of body weight of *S. riddellii*.	An increase in connective tissue thickening around bile ducts and blood vessels: no true cirrhosis.
168	7–14	(Did not die)	True cirrhosis.
170	5–5	Before fed any green Senecio.	Poor specimen but appears normal.
170	5–26	After being fed 9.8 % of body weight of *S. longilobus*.	True cirrhosis in some areas with increase in fibroblasts around all small vessels.
170	6–15	Dead.	Autopsy—cirrhosis with yellow icteric liver.

The same was not true of calf 170 since he died sooner than expected. For that reason, liver biopsy would have been of very little practical value in his case. The more chronic cases, which are the types commonly seen on the range, can be diagnosed by liver biopsy.

Ninety-eight liver biopsies were taken from normal-appearing adult animals. These biopsies were sectioned by the paraffin method, stained, and examined microscopically. Sixty-nine of these specimens were found to be essentially normal. The others had some liver damage, ranging from slight cloudy swelling to well-marked fibrosis. None showed a true cirrhosis.

By June 18, when the last liver samples were taken, the procedure could be speeded up considerably if heavy duty Oster clippers were used to prepare the operative site. A vibrator converter was used to change the battery current from 6 volts to 110 volts. Using this equipment, one sample was taken in approximately ten minutes. With a good squeeze chute and a crew of four men to handle the animals, at least 75 animals could be operated upon in an eight-hour day. This might be increased if two squeeze chutes were available. The animals would have to be rehandled and the ear tags of those showing liver cirrhosis identified after the tissues are examined; however, on ranches where losses were occurring, this procedure would be practical.

SUMMARY AND CONCLUSIONS

The bromsulfalein liver function test was run on 8 normal cattle and on 5 experimentally produced cases of Senecio poisoning—before the Senecio plants were fed, during the feeding of the plants, and after signs of poisoning developed. A total of 15 tests were made on 2 other calves which were fed Senecio silage over a long period.

Experience indicates that this test is dependable, as a means of diagnosis, only in

the last stages of chronic cirrhosis of the liver. The test indicated impaired liver function in some instances when microscopic examination of liver biopsies did not show cirrhosis. Thus, this test is considered too sensitive for practical use. It is impractical under range conditions because it consumes too much time and, in most instances, does not differentiate between acute hepatitis and chronic cirrhosis. Many of the range cows in which biopsy revealed some microscopic changes would undoubtedly have shown a loss in liver function by this test. The liver regeneration and compensation apparently overcomes the acute hepatitis and unless a true cirrhosis is present, such cows never show any signs of poisoning.

Liver biopsies on 117 cattle were taken by the use of Wall's hepatic trochar. Some were from experimentally produced cases of Senecio poisoning and some from animals fed Senecio silage. Forty-eight were taken under range conditions from apparently normal cattle.

Indications from this work are that this liver biopsy procedure, as adapted by this station, is a good method of diagnosing cirrhosis of the liver in live cattle.

The entire procedure is simple, is not dangerous to the animals and is reliable as a method of diagnosis.

References

[1] Mateer, John G., Baltz, James I., Marion, Donald F., MacMillan, J. M.: Liver Function Tests. A General Evaluation of Liver Function Tests and an Appraisal of Comparative Sensitivity and Reliability of the Newer Tests with Particular Emphasis on Cephalin Cholesterol Flocculation Tests, the Intravenous Hippuric Acid Test, and an Improved Bromsulfalein Test with a New Normal Standard. J. Am. M. A., *121*, (March 6, 1943): 723-728.

[2] Isaksson, Axel: Wall's Liver Biopsy for Horses. J.A.V.M.A., *118*, (1951): 320-322.

Inflammatory Response of the Bovine Endometrium

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LITERATURE CITED

Tanabe [10] in 1947 observed pyometra in some previously unbred heifers treated with gonadotrophins and inseminated during the luteal phase of the estrous cycle. Work was then transferred to sows where a somewhat similar phenomenon in the luteal, but not in the follicular phase, was noted.[16] Willett *et al.*[17, 18] obtained confirmatory evidence for this differential behavior in superovulated heifers.

Pyometra was found almost invariably in pseudopregnant rabbits following deposition of semen in their uteri;[2, 3] estrous females so treated did not demonstrate this same inflammatory response. In a comparison of rabbit semen ejaculated into an artificial vagina and epididymal rabbit sperm collected free of bacteria,[3] it was suggested that the causative agent in the production of pyometra was in all probability bacteria contaminating the ejaculated semen. Further studies [2] demonstrated that certain bacteria could indeed produce this condition. Teunissen,[16] in intact and spayed bitches treated with estrogen and progesterone, demonstrated an association between endometritis and the gonadal hormones. Further, in studies of ovariectomized rabbits treated with such hormones,[3] it was shown that the presence or absence of the inflammatory response was associated with the presence or absence of effective levels of progesterone.

These investigations suggest that the response of the mammalian uterus to bacteria varies with the stage of the reproductive cycle. In view of certain practices now in use in the bovine female (artificial insemination, uterine irrigation, etc.) which involve invasion of the uterus, it would seem important to determine the consequences of such procedures in the light of possible bacterial infection.

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One aspect of the present study has been concerned with lowered fertility of otherwise clinically normal cows. Several workers have suggested that this condition might be accounted for by low grade infections of the reproductive organs. A detectable difference between normal and repeat-breeding cows in the inflammatory response (to the extent that this response may reflect resistance or susceptibility to bacterial infection) might suggest an endocrine dysfunction as the underlying cause of lowered fertility.

MATERIALS AND METHODS

Ten virgin heifers and 18 mature cows, clinically normal "repeat breeders," were used in these studies. Criteria used in the selection of the repeat-breeding animals and something of the nature of the infertility have been described by Tanabe and Casida [14] and Christian *et al.*[5]

The general procedure followed in the study of these animals was to inseminate the individuals at the time of heat or at various stated periods following heat. The semen * was yolk-citrate diluted as used in field practice. Sulfonamides and antibiotics were diluter constituents of most samples. The techniques used in insemination have varied from trial to trial and are described later. Individuals were slaughtered twenty-four to thirty-six hours following insemination and the reproductive organs recovered. The uteri were opened and the degree of response to the inoculum noted grossly. Sections of the horns and body of the uterus of some animals were saved in Mossman's fixative and stained with hematoxylin and eosin for histological study.

RESULTS

Differential Response to Semen in Virgin Heifers.—In nulliparous cattle, the cervix during the luteal phase of the estrous cycle is generally so constricted as to make difficult the passage of an inseminating tube into the uterus without some trauma. To make a direct comparison of the inflammatory response to semen in both estrous and luteal-phase heifers without this complication, it was decided to avoid the cervical canal and perform a

* Supplied by Southern Wisconsin Breeders Coöperative, Madison, Wis.

midventral laparotomy to inject the uterus. One milliliter of semen was introduced into the lumen of one horn by puncturing the uterine wall at the level of the external bifurcation with a sterile hypodermic needle and syringe.

Three heifers were inseminated in this manner approximately twelve hours after first noticing heat. Three other animals were treated similarly twelve days after the day of heat. A gross reaction was not observed in any of the heifers inseminated during the follicular phase. The uteri of 2 animals inseminated during the luteal phase contained a thick, creamy, light yellow pus. Estimates of the volume of this material were of the order of 3 to 5 ml. The uterus of the third luteal-phase heifer, while manifesting no gross reaction, contained a small quantity of thin, dark fluid.

Semen as usually collected and handled is rarely found to be free of bacteria. Addition of antibiotics and sulfonamides to the diluter usually prevents further growth of these organisms and may destroy all contaminants.[1,6] However, passage of an inseminating tube through the vagina and cervix, regardless of the customary precautions observed in field practice, is very likely to introduce further contaminants into the uterus. The technique used in the present trial, however, should be one in which this source of contamination is not a factor. The pyometra observed following insemination during the luteal phase is believed to be the result of bacterial contamination of the semen itself, although the response of the uterus to the diluter constituents, particularly egg yolk, has not been studied. Failure to obtain a response from 1 luteal-phase heifer may lie in the use of a semen sample free or relatively free from bacterial contamination, but since some difficulty was experienced in verifying penetration into the uterine lumen by the hypodermic needle, failure may also have been the result of defective technique.

Differential Response of the Heifer and Repeat-Breeding Adult.—The comparison of the uterine reaction of heifers and repeat-breeding cows in the luteal phase of the estrous cycle was an outgrowth of some preliminary efforts to modify the reactions in the latter animal by use of hormones as had been done in rabbits.[3] Two repeat-breeding cows treated with subcutaneous injections of 20 mg. of estradiol propionate on days 10 and 12 of the estrous cycle and then inseminated via the cervix into the uterus on day 13 did not react. Two other repeat breeders treated with progesterone and then inseminated into the uterus at estrus, however, also failed to react. One of these received 100 mg. of progesterone in oil subcutaneously when first noticed in heat; the other received 12.5 mg. daily from day 15 until heat was observed (day 25). This raised the question whether the reaction in the first animals was actually being prevented by the estrogen or whether repeat-breeding cows do not respond as do heifers. Consequently, 6 other repeat breeders were uterine-inseminated through the cervix eight to twelve days after heat and the uterine conditions then studied. These animals demonstrated extreme variability in response (table 1). Two evidenced no gross reaction while the lumen debris in the uteri of the other 4 ranged from a slight amount of thin, milky fluid (probably not a reaction) to as much as 4 to 5 ml. of thick, coagulated pus. This variability in response might be accounted for by variability in the degree of semen contamination, but 2 additional cows were inseminated with rabbit semen (known to be highly contaminated[6]) and in only 1 was typical pyometra obtained. A further repeat breeder inoculated *in utero* with a suspension of *Escherichia coli* organisms demonstrated typical but not extensive pyometra.

A direct comparison was next carried out utilizing virgin heifers and repeat breeders. To eliminate variation in the de-

TABLE 1—Uterine Response in the Mature Cow (Repeat Breeder)

Cow (No.)	Uterine inoculation (days postestrus)	Inoculum	Pus in uterine lumen
80	12	Bovine semen	None
87	12	Bovine semen	1-2 ml.
85	12	Bovine semen	None
7	12	Bovine semen	1 ml.
97	11	Bovine semen	4-5 ml.
94	8	Bovine semen	None*
88	12	Rabbit semen	2 ml.
98	12	Rabbit semen	Barely perceptible
19	10	*Escherichia coli* suspension	1 ml.

* Milky fluid barely perceptible.

TABLE 2—Differential Response of Heifer and Cow (Repeat Breeder) to Semen

	Heifer			Cow		
Semen sample	Animal (No.)	Insemination (days postestrus)	Pus in uterine lumen (ml.)	Animal (No.)	Insemination (days postestrus)	Pus in uterine lumen (ml.)
Pr	46	7	3.0	3	8	None
Pi	68	13	4.0	15	11	None
Le	63	10	0.5*	17	12	None
Ad	81	11	1.0	18	12	None**

* May have been mucus containing yolk from diluter.
** Slight amount of clear mucus, reddish yellow.

gree of contamination of the semen, 1 heifer and 1 cow were inseminated simultaneously with the same sample. To facilitate matching of heifer and cow, it was decided to permit as much as seven days' difference in the time after heat in which insemination was to occur, providing that both animals were treated in the interval seven to fourteen days postestrum. A Woelffer uterine catheter was used to permit passage through the cervix more readily. Approximately 2 ml. of semen were deposited in the right uterine horns.

Four heifer-cow pairs were treated. All heifers exhibited some lumen material at slaughter, varying from thick, yellow mucus to definite puslike debris (table 2). Three of the cow uteri, on the other hand, were free of any material; the fourth animal showed a clear, reddish yellow mucus. These gross observations fail to differentiate accurately between a true pyometra and mucus containing residual egg yolk of the semen diluter. Histological studies of these uteri, however, revealed in general an acute endometritis accompanied by pyometra in the heifers. The repeat-breeding cows, on the other hand, showed considerably less cellular exudate, and the subepithelial invasion in these uteri consisted of greater proportions of lymphocytes and mast and plasma cells, rather than polymorphonuclear leukocytes. The general impression derived from these microscopic studies was that in the heifer the response was of an acute nature, while in the repeat-breeding cow the reaction was of a more chronic character, as if this uterus had been subjected frequently to such stimuli. Again, it must be borne in mind that this comparison is one between a first-service heifer and a repeat-breeding cow, rather than between normal and low fertility in cows of similar ages.

DISCUSSION

The present studies verify the work of earlier investigators that the luteal-phase uterus is more susceptible to instilled material than that of the follicular-phase animal. It is likewise probable that bacteria constitute the irritant-stimulus leading to pyometra in these experimental animals, as is the case in the rabbit. These findings in themselves are sufficient to warrant use of extensive prophylactic measures to avoid bacterial infection whenever the uterus must be invaded in large animal practice. Rowson [12] and Lamming and Rowson,[7] in the course of embryo transplantation studies, observed pyometra in most cows following insemination during the luteal phase, and were able to induce infection in other postestrous animals following introduction of tubal washings containing penicillin. They have, therefore, postulated that such infection may be the main barrier to successful transplantation via the cervix. Since this technique must be applied during the luteal phase, any bacterial infection may lead to an inflammatory response which, in turn, would interfere with placentation. Bacteria introduced in this manner may also attack the embryo directly. Evidence that this phenomenon may actually occur was presented by Black *et al.*[2] where, in artificially ovulated pseudopregnant rabbits, all embryos resulting from uterine insemination died prior to the fifteenth day of gestation.

The observations made here seem to indicate that the repeat-breeding animal does not behave in a manner similar to that of the nulliparous heifer. Some variability in response to semen has also been noted in the heifer. Tanabe [13] found a pronounced pyometra in 3 of 5 heifers treated with gonadotrophins and inseminated during the luteal phase (seven to nine days postestrus) when slaughtered forty-five to sixty-one hours later. The uteri of the remaining 2 animals contained only relatively clear mucus. Willett *et al.*[17,18] observed pyometra in all of 6 heifers inseminated nine and ten days after the last heat and killed three or five days later. However, a greater proportion of repeat breeders demonstrate no observable response,

and in those cases where a reaction is found, it is generally less extensive.

Studies of repeat-breeding cows [14] indicate that early embryonic mortality is a major component of the infertility observed. The time at which the majority of these embryos die is not at present known, but since estrous cycle lengths in such cows are often not unduly long, death of the embryo and regression of the corpus luteum must usually occur within the first sixteen to eighteen days after the "infertile" service. This perhaps suggests failure in the early placentation of the blastocyst. A deficiency of progesterone may lead to an endometrium unsuitable for proper nourishment of the young embryo, or would prohibit placentation. The observation that the uterus of the repeat-breeding cow in the luteal phase of the estrous cycle does not react to insemination to the same degree as does the uterus of the heifer may suggest such a progesterone deficiency.

A further possible cause of such infertility is suggested by the relative nonresponsiveness of the repeat breeder. Some mechanism must exist whereby the ovary and corpus luteum is physiologically aware that an embryo is developing within the uterus, such that the corpus luteum continues to function and supply the necessary progesterone to the endometrium. The stimulus which activates this mechanism must lie within the embryo itself. If this stimulus consists of some form of irritation of the endometrium, then the nonirritability of the repeat-breeder endometrium, as typified by failure in demonstrating an inflammatory response to semen, may also prevent transmission of the embryonic stimulus to the corpus luteum.

Other possibilities exist that might account for the lowered fertility observed in this class of animals. If it is assumed that absence of pyometra in the estrous uterus indicates the existence of an effective defense against bacterial infection, it is conceivable that a high level of progesterone in the circulation at the time of estrous (owing to retarded regression of the corpus luteum) may reduce the efficiency of this mechanism and permit bacterial contaminants to gain a foothold in the uterus. These bacteria might then be in position to attack the young embryo or otherwise interfere in its development. Evidence for the existence of such organisms is suggested by the work of Rottensten [10,11] and Christian and Casida.[4] These investigators observed that certain bulls appeared to affect the later fertility of cows to which they were bred. These particular bulls may transmit in their semen certain organisms of low grade pathogenicity. In some of these recipients, this infection may not be eliminated during the period in which the natural defense is operating at its greatest efficiency, but may persist and become established in the uterus, there to be in position to interfere with fertility of the subsequent service.

SUMMARY

The development of a pyometra has been compared in follicular and luteal-phase heifers, and in "first-service" heifers and "repeat-breeder" cows both in the luteal phase of the estrous cycle.

Yolk-citrate-diluted bovine semen was deposited in the uteri of 3 estrous and 3 twelve-day luteal-phase virgin heifers by laparotomy and hypodemic puncture of the uterine wall. No response was obtained in the estrous females at slaughter twenty-four to thirty-six hours later. The uteri of 2 of the luteal-phase heifers contained pus; the response in the third animal was not typical.

Semen was deposited in the uteri of 6 repeat-breeding cows eight to twelve days after heat by cervical insemination. The degree of response seen in their uteri at the time of slaughter was highly variable, ranging from no gross reaction in 3 to as much as 4 to 5 ml. of pus in others. Response to rabbit semen in 2 other cows was likewise variable.

Comparisons were made of the response to identical semen samples in paired luteal-phase virgin heifers and repeat-breeding cows. Pus was found in none of the uteri of 4 cows, while 3 of the corresponding heifers demonstrated pyometra; the response in the fourth heifer was doubtful in nature.

These results suggest that the repeat-breeding cow does not respond to insemination during the luteal phase of the estrous cycle in the same manner as does the virgin heifer. The relative nonresponsiveness seen

in the cows may indicate an endocrine dysfunction underlying their lowered fertility.

References

[1] Almquist, J. O., Glantz, P. J., and Shaffer, H. G.: The Effect of a Combination of Penicillin and Streptomycin upon the Livability and Bacterial Content of Bovine Semen. J. Dai. Sci., *32*, (1949): 183.

[2] Black, W. G., Otto, G., and Casida, L. E.: Embryonic Mortality in Pregnancies Induced in Rabbits of Different Reproductive Stages. Endocrinology, *49*, (1951): 237.

[3] Black, W. G., Simon, J., McNutt, S. H., and Casida, L. E.: Investigations of the Physiological Basis for the Differential Response of Estrous and Pseudopregnant Rabbit Uteri to Induced Infection. Am. J. Vet. Res., *14*, (1953): 312-317.

[4] Christian, R. E., and Casida, L. E.: The Effects of Infertile Insemination and Individuality of Bulls upon the Subsequent Fertility of Cows Returning for Service. J. Dai. Sci., *34*, (1951): 971.

[5] Christian, R. E., Ulberg, L. C., Phillips, P. H., and Casida, L. E.: The Response of Low-Fertility Cows to Chlorobutanol and Ascorbic Acid Administration. J. Dai. Sci., *34*, (1951): 978.

[6] Foote, R. H., and Bratton, R. W.: Motility of Bovine Spermatozoa and Control of Bacteria at 5 C. and 25 C. in Extenders Containing Sulfanilamide, Penicillin, Streptomycin and Polymyxin. J. Dai. Sci., *33*, (1950): 842.

[7] Lamming, G. E., and Rowson, L. E. A.: Superovulation and Ovum Transplantation in Cattle. Rep., Second Internat. Congress of Physiol. and Path. of Animal Reproduction and of Artificial Insemination, *1*, (1952): 144.

[8] McDonald, L. E., Black, W. G., McNutt, S. H., and Casida, L. E.: The Response of the Rabbit Uterus to Instillation of Semen at Different Phases of the Estrous Cycle. Am. J. Vet. Res., *13*, (1952): 419.

[9] Murphree, R. L., Black, W. G., Otto, G., and Casida, L. E.: Effect of Site of Insemination upon the Fertility of Gonadotrophin-Treated Rabbits of Different Reproductive Stages. Endocrinology, *49*, (1951): 474.

[10] Rottensten, K.: Iagttagelser og Undersøgelser over Nedsat Frugtbarhed hos Kvaeg. 235 Beretn. fra Forsøgslaboratoriet, København.

[11] Rottensten, K.: Iagttagelser og Undersøgelser over Nedsat Frugtbarhed hos Kvaeg. II. 247 Beretn. fra Forsøgslaboratoriet, København.

[12] Rowson, L. E.: Methods of Inducing Multiple Ovulation in Cattle. J. Endocrin., *7*, (1951): 260.

[13] Tanabe, T. Y.: Unpublished data. 1947.

[14] Tanabe, T. Y., and Casida, L. E.: The Nature of Reproductive Failures in Cows of Low Fertility. J. Dai. Sci., *32*, (1949): 237.

[15] Tanabe, T. Y., Warnick, A. C., Casida, L. E., and Grummer, R. H.: The Effects of Gonadotrophins Administered to Sows and Gilts During Different Stages of the Estrual Cycle. J. Anim. Sci., *8*, (1949): 550.

[16] Teunissen, G. H. B.: The Development of Endometritis in the Dog and the Effect of Oestradiol and Progesterone on the Uterus. Acta Endocrin., *9*, (1952): 407.

[17] Willett, E. L., McShan, W. H., and Meyer, R. K.: Relation of Source of Hormone, Dosage, and Stage of Cycle to Superovulation in Heifers. J. Anim. Sci., *7*, (1948): 545 (abstr.).

[18] Willett, E. L., McShan, W. H., and Meyer, R. K.: Relation of Stage of Cycle and Source of Luteinizing Hormone to Superovulation in Dairy Cattle. Proc. Soc. Expti. Biol. and Med., *79*, (1952): 396.

Acute and Prolonged Insulin Hypoglycemia in Cows

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HYPOGLYCEMIA accompanies primary ketosis of cows and, though often severe, it may at times be only moderate or slight.[20, 21, 23-25] A study of these and other reports reveals also that a definite inverse relationship usually exists between blood sugar and blood ketone levels.

In general, the severity of symptoms displayed is fairly well correlated with the degree of hypoglycemia and/or ketonemia, but many exceptions have been observed.[12] The seriousness of the symptomatology, therefore, does not appear to be a direct function of either quantity without some modifying factor. As pointed out by Fisher,[7] there is much direct evidence both supporting and opposing the hypothesis of primary ketone toxicity. The present investigation was undertaken to demonstrate the role hypoglycemia may play in the symptomatology of ketosis.

It has long been known that hypoglycemia, as a result of insulin injection or from other causes, produces rather characteristic symptoms in man and in the more common laboratory animals such as the dog, cat, rat, mouse, and rabbit. As pointed out by Reid,[17] definite hypoglycemic signs occur after 1 to 2 units of insulin per kilogram and injection of 2 to 3 units is usually followed by convulsions. That convulsions are not inevitable at the latter levels of insulin injection in dogs and cats was demonstrated in Reid's review of the literature, e.g., Dworkin[6] stating that as much as 3 to 5 units per kilogram were necessary. Zucker and Berg[28] observed that increased dosage of insulin was reflected almost exclusively by increased duration of action in the dog, whereas in the cat both duration and intensity are increased.

The effects of insulin injection in monogastric animals appear to be reasonably uniform and well known, but in the few reports[1-5, 10, 11, 14, 15, 22, 26, 28] of insulin injections into ruminants the results have been variable and inconclusive until the very recent work on sheep published by Reid.[17, 18]

Although the effects of insulin hypoglycemia in cows is not clear from these reports, the assumption of Roepke[19] that nervous symptoms of ketosis were due to hypoglycemia is logical in view of the fact that nerve cells are thought to use only glucose for purposes of energy.[9] Explanation of ketosis symptoms on this basis has been complicated by the severe spontaneous and experimental hypoglycemias sometimes observed without obvious symptoms. In such cases, however, it appeared that the duration of the asymptomatic hypoglycemias may be much shorter than is the case in clinical ketosis. If this is true, the time factor may be important in eliciting nervous symptoms from hypoglycemia in the cow.

These experiments were designed to determine (1) the effect of single injections of insulin at various levels upon depth and duration of hypoglycemia and (2) the clinical symptoms resulting from various depths and durations of hypoglycemia.

METHODS

Hypoglycemia was produced by intravenous or subcutaneous injection of regular insulin (Lilly) and maintained by subcutaneous injections of protamine zinc insulin (Lilly). True blood sugar values were determined either by the method of Nelson[14] or by the Folin-Malmros[8] method using a zinc sulfate filtrate. Sodium fluoride was added to potassium oxalate tubes to prevent glycolysis prior to precipitation. All blood sampling and intravenous injections were made via the jugular vein. Determinations were made in duplicate, and all very low values and cases of disagreement were rechecked in duplicate. Larger aliquots of blood

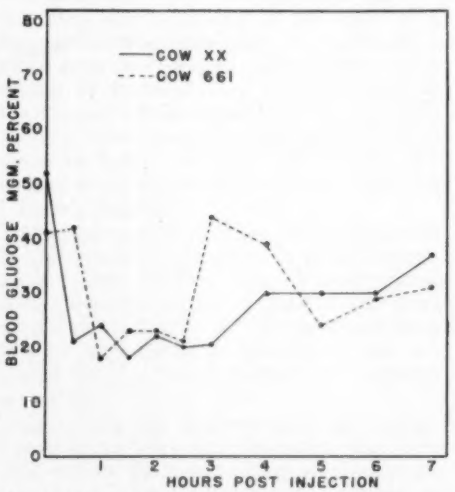

Fig. 1—Hypoglycemia from 1 unit of insulin per kilogram intravenously.

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The data from cows 700 and 703 were taken from a thesis submitted in 1947 in partial fulfillment of the requirements for the degree of doctor of philosophy at the University of Minnesota. The counsel and help of Dr. M. H. Roepke at that time is gratefully acknowledged.

and filtrate were often used in case of samples with low values in order to keep the galvanometer readings within a more accurate range.

RESULTS

The Effect of Increasing Dosages of Regular Insulin Given Intravenously.—Two nonpregnant cows were used for these observations. One cow, 661, a Holstein-Friesian, was furnishing small amounts of milk to a calf except during the course of the experiments. Cow xx, a grade Guernsey, was nonlactating. A week or more elapsed between experiments with the exception of a second trial after two days on cow 661 using 2 units of insulin per kilogram.

The resultant course of the blood sugar levels is plotted in figures 1, 2, 3, and 4. It is readily seen that the effect upon duration of hypoglycemia varied more regularly with changes in dosage than did depth of hypoglycemia. If a state of hypoglycemia is considered to be present when the blood sugar is below 20 mg. per 100 cc. of blood, the duration of hypoglycemia was noted to be one and one-half to two and one-half hours following injection of 1 mg. per kilogram of regular insulin intravenously. With doses of 2, 5, and 10 mg. per kilogram respectively, the duration of hypoglycemia was about four to five hours, seven to eight, or more, and twelve hours.

Definite hypoglycemic symptoms in cow 661 were seen between one and eight hours after she received 5 units of insulin per kilogram. Signs were present in cow xx between two and eight hours after injection of 2 units of insulin per kilogram and between two and fourteen hours after injection of 10 units per kilogram. Signs consisted of dullness, refusal of proffered feed, cessation of bowel movements, or passage of only small amounts of firm feces, and, in the case of cow xx, after 2 units per kilogram, muscular tremors.

The Effect of Prolonged Insulin Hypoglycemia in Cows.—Prolonged insulin hypoglycemia was produced by injection of

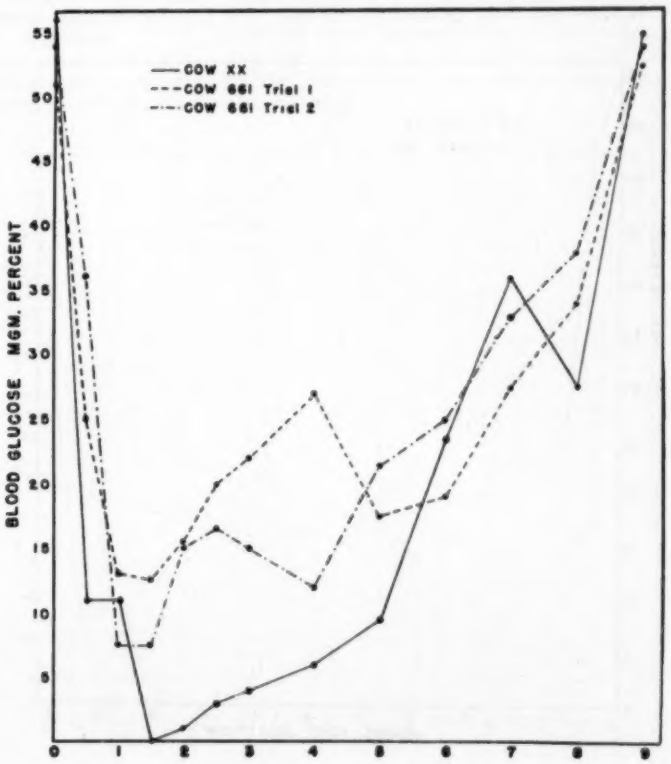

Fig. 2—Hypoglycemia from 2 units of insulin per kilogram intravenously.

regular insulin subcutaneously in cows 700 and 703, and intravenously in cows xx and 661. Protamine zinc insulin administered subcutaneously was used to maintain hypoglycemia. The resulting hypoglycemias are recorded in figures 5, 6, and 7. In figures 6 and 7, the relative intensity of observed symptoms was plotted in an arbitrary manner in order to give a visual picture. All cows were nonpregnant. Cows 661, 700, and 703 were lactating lightly.

Reid[16] divided the syndrome in sheep into four stages. The signs observed in the cows varied somewhat in detail but were similar in nature to those recorded in sheep. In addition, definite individual differences were noted, some signs being especially marked at times and in other cases being entirely absent. For purposes of discussion, the symptoms observed in these cows will be divided into three stages.

Stage 1.—Depression and lethargy were the most constant signs, though nervousness was sometimes observed. Muscular tremors of the triceps region or of the head and neck were common. The head was frequently held low and the gait became a bit awkward. Poor prehension sometimes appeared at this time. Hypersalivation was constant, sometimes accompanied by lacrimation. Hyperesthesia was common and in 1 case photophobia was observed. Feces became dry and scanty in amount. Anorexia was not constant in this stage.

Symptoms became obvious about twenty hours postinjection. Transitory or very mild symptoms such as dullness or tremors were observed in 2 cases as early as three or four hours postinjection. Blood glucose levels were somewhat variable but were hypoglycemic most of the time as may be seen by reference to the figures. This stage persisted approximately ten to twenty-five hours during induction stages. In cow xx, which received no glucose, this stage returned again for twenty hours following stage 2 symptoms.

Stage 2.—Usually the symptoms of stage 1 became more marked and, in addition, the gait became very unsteady and staggering. The animal at times attempted but failed such activities as eating hay or licking her side. Anorexia was common but not always present. Hypersalivation was often marked but was sometimes absent. In all but animal 700, which passed through this stage very hurriedly, very pronounced continuous chewing or sucking motions persisted for hours. Feces were firm and formed, little or none being passed. Cutaneous

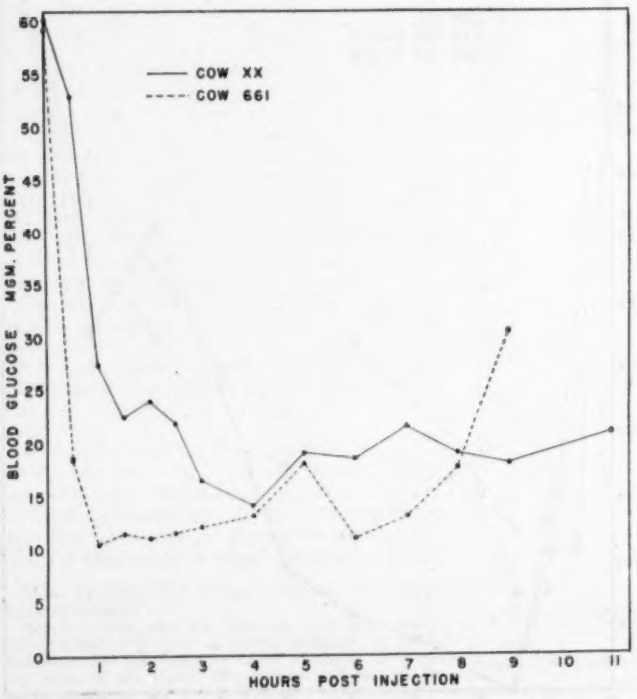

Fig. 3—Hypoglycemia from 5 units of insulin per kilogram intravenously.

hyperesthesia, manifested by biting or licking certain areas, was marked in 2 cows. Cow xx remained in this stage for sixteen hours with blood glucose levels varying between 13 and 22 mg. per 100 cc. of blood, after which time symptoms abated. In cow 661, stage 2, symptoms persisted for four hours at glucose levels between 5 and 18 mg. per 100 cc. of blood and in cow 703 they lasted six hours at glucose levels between 8 and 21 mg. per 100 cc. of blood. In each case, stage 2 signs appeared after twenty-six to thirty hours of hypoglycemia.

Stage 3.—The most characteristic symptoms of this stage were inability to stand or to rise and marked incoördination. Sucking or licking activities were sometimes exaggerated. Respiration was at times gasping, irregular, and accompanied by moaning. In the absence of physical activity, the posture was suggestive of that seen in cases of parturient paresis. The behavior suggested near coma or near convulsions, but neither state was clearly present. Symptoms were alarming enough however, in cows 700 and 703, to prompt immediate administration of glucose. The almost convulsive aspects of stage 3 in cows 700 and 703 were noted after thirty-three and thirty-five hours of hypoglycemia. The last glucose levels recorded before the intensive seizures being 14 and 21 mg. per 100 cc. of blood, respectively.

Cow 661 first went down after about thirty hours of hypoglycemia, the symptoms gradually becoming more severe during the next six hours at which time 250 ml. of 50 per cent glucose were given. Blood glucose levels recorded during this period of marked symptoms varied between 11.5 and 15.5 mg. per 100 cc. of blood.

Administration of glucose to cows 661, 700, and 703 when in the near convulsive state was followed by prompt remission of uncontrolled or meaningless physical activity, resulting in a state of quiet and rest. Cow 661 suffered a slight relapse as the blood sugar dropped again to 5.5 mg. per 100 cc. of blood.

Symptomatically, complete normality was regained eighteen, thirty-six, and fifty-seven hours after glucose in cows 661, 700, and 701. The longer times observed in cows 700 and 701 were due chiefly to delay in the return of normal locomotion, especially in the hind quarters. Cow 703 displayed a rather persistent limp in the left hind leg. Cow 700 stepped high with the hind legs as if stepping over objects while cow 661 walked, for a time, with an action simulating "stringhalt."

Discussion

As in the case of sheep,[17] the response of cows to single injections of insulin is probably subject to considerable variation, depending upon the condition of the animal and the nature of the ration. Individual animal variations from time to time may also be large.

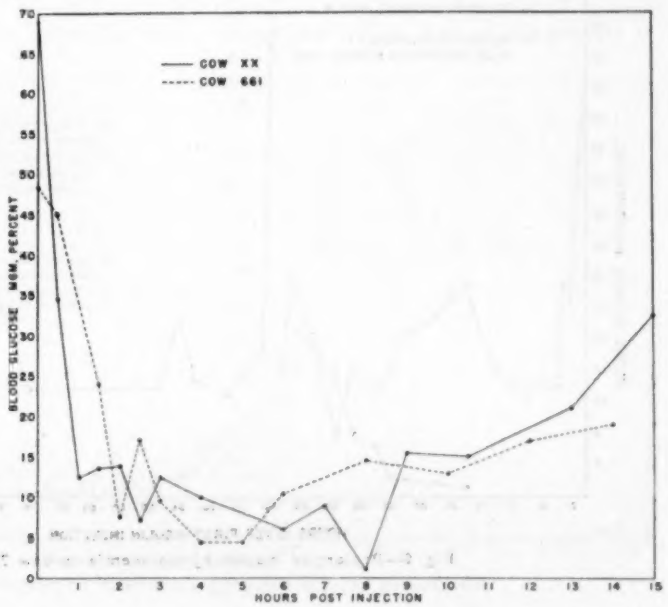

Fig. 4—Hypoglycemia from 10 units of insulin per kilogram intravenously.

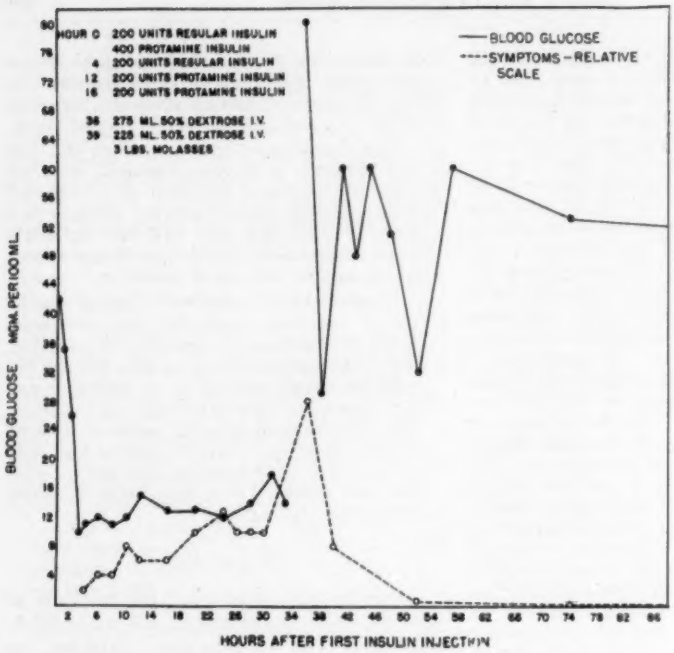
HOUR 0 200 UNITS REGULAR INSULIN
400 PROTAMINE INSULIN
4 200 UNITS REGULAR INSULIN
12 200 UNITS PROTAMINE INSULIN
16 200 UNITS PROTAMINE INSULIN
36 275 ML. 50% DEXTROSE I.V.
39 225 ML. 50% DEXTROSE I.V.
3 LBS. MOLASSES
BLOOD GLUCOSE
SYMPTOMS – RELATIVE SCALE
BLOOD GLUCOSE MGM. PER 100 ML.
HOURS AFTER FIRST INSULIN INJECTION

Fig. 5—Prolonged insulin hypoglycemia in cow 700.

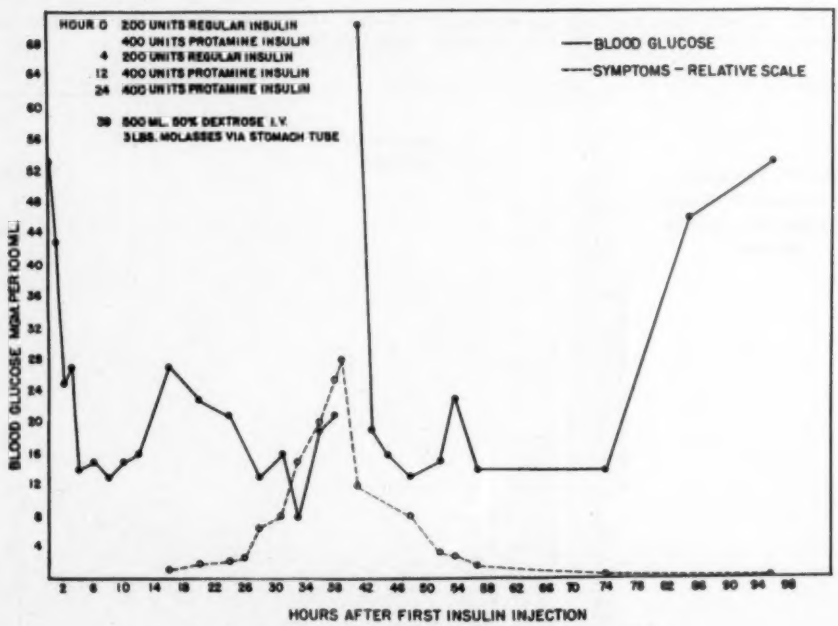
HOUR 0 200 UNITS REGULAR INSULIN
400 UNITS PROTAMINE INSULIN
4 200 UNITS REGULAR INSULIN
12 400 UNITS PROTAMINE INSULIN
24 400 UNITS PROTAMINE INSULIN
39 500 ML. 50% DEXTROSE I.V.
3 LBS. MOLASSES VIA STOMACH TUBE
BLOOD GLUCOSE
SYMPTOMS – RELATIVE SCALE
BLOOD GLUCOSE MGM. PER 100 ML.
HOURS AFTER FIRST INSULIN INJECTION

Fig. 6—Prolonged insulin hypoglycemia in cow 703.

One unit of insulin per kilogram did not appear to give maximum hypoglycemia. Increasing the dosage beyond 2 units per kilogram was not followed by greater hypoglycemia. Indeed, the lowest recorded level, 0.0 mg. per 100 cc. of blood followed injection of only 2 units per kilogram. In each curve, a slight rebound or increase in blood glucose followed the primary descent to a hypoglycemic level. The original hypoglycemic level, or perhaps an even lower level, was usually quickly reëstablished. This intermediate rise may be of the same nature as that observed to occur in dogs twenty to thirty minutes following intravenous injections of insulin.[28]

Considerable variation was encountered in the time elapsing between injection of insulin and initiation of the fall in blood glucose. This delay was probably the result of individual variations in the state of metabolism at the time of injection rather than a factor dependent upon size of dose, since delay was noted after injection of 10 units per kilogram as well as after smaller doses. Both animals showed delay but following different amounts of insulin. Reid[17] observed a marked delay in onset of hypoglycemia following injection of insulin in 6 of 9 ewes during the last two months of pregnancy and a rise of 4 to 6 mg. per 100 cc. of blood after thirty minutes in 3 of these. A rise of 1 mg. per 100 cc. of blood after thirty minutes was noted in cow 661 after 1 unit per kilogram of insulin. If cows show a normal oscillation as do dogs,[28] the delay may possibly be partially explained on this basis.

The rate of decrease may appear at first glance to be highly variable, especially if considered in terms of fall in milligrams per 100 cc. of blood thirty minutes after insulin. Such an expression would result in a wide range of values between —1.0 and 45.0 mg. per 100 cc. of blood. Examination of the curves indicates, however, that the rate of fall, as demonstrated by the slope of the curve, was not greatly different once the fall had begun. Critical determination of rate of fall of blood glucose can not be made accurately from these studies, because sampling was not sufficiently frequent to ascertain the exact time limits of the fall period. Selections of thirty-minute periods from the curves yielded falls of between 22 and 45 mg. per 100 cc. of blood. It is probable, however, that in cases where lower rates were noted, such as in the thirty- to sixty-minute period with cow xx in figure 4, the fall had stopped some minutes before the sample was taken. A rebound may even have started. Most of these slopes indicated a fall of 25 to 30 mg. per 100 cc. of blood in thirty minutes, although a fall of at least 40 mg. per 100 cc. of blood was present in

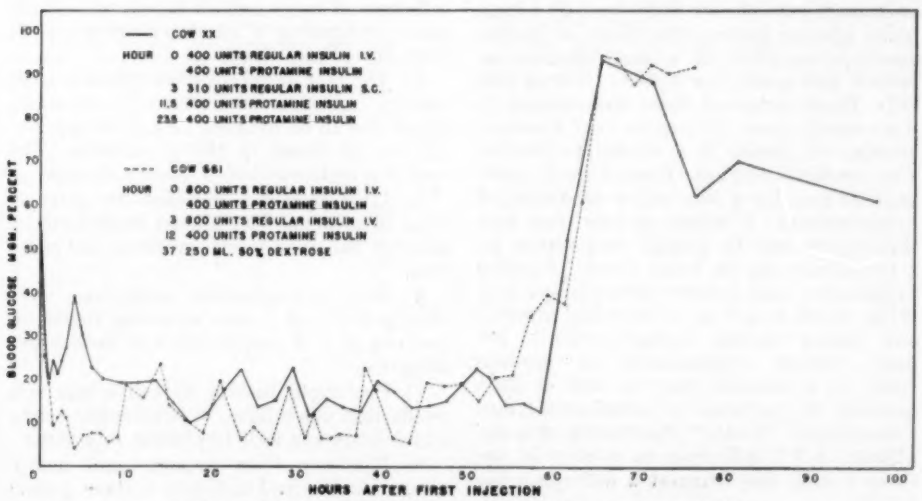
COW XX
HOUR 0 400 UNITS REGULAR INSULIN I.V.
400 UNITS PROTAMINE INSULIN
3 310 UNITS REGULAR INSULIN S.C.
11.5 400 UNITS PROTAMINE INSULIN
23.5 400 UNITS PROTAMINE INSULIN
COW 661
HOUR 0 800 UNITS REGULAR INSULIN I.V.
400 UNITS PROTAMINE INSULIN
3 800 UNITS REGULAR INSULIN I.V.
12 400 UNITS PROTAMINE INSULIN
37 250 ML. 50% DEXTROSE
BLOOD GLUCOSE MGM. PERCENT
HOURS AFTER FIRST INJECTION

Fig. 7—Prolonged insulin hypoglycemia in cows xx and 661.

2 cases. The rate of fall did not appear to be correlated with dosage or initial blood glucose level. These results indicate a rate of fall of blood glucose in cows well below that of 50 to 60 mg. per 100 cc. within twenty to thirty minutes as found in man, rabbits, and the dog but greater than mean rates of 17.1 to 24.7 for this dosage range in sheep.[17]

Experimental prolonged hypoglycemia in cows also demonstrates a reaction different from that usually observed in nonruminants. Production of well-defined symptoms may be delayed for several hours following production of hypoglycemia. The development of serious signs was more closely correlated with duration than with depth of hypoglycemia. In each case, a crisis characterized by convulsions, or near convulsions, occurred thirty-three to thirty-six hours after initial hypoglycemia. In cows 700 and 703, the general trend of the glucose level at the time of crisis was above that of a few hours before when much less severe symptoms were shown.

In cow 703, the hypoglycemic effect of injected protamine insulin remained for some time after the hypoglycemic crisis. Actually, with one exception, the blood glucose levels were recorded at about 14 mg. per 100 cc. of blood for over thirty hours following the crisis. In spite of this, the animal returned to normal at the approximate rate of cow 700 who had a high blood glucose during this time. A similar ameliorating effect of a small glucose injection was noted to a lesser extent in cow 661. Explanation of these observations is not entirely clear. It may be that a certain amount of glucose is available to the bovine cerebral neurons through some intermediate pool for a time under conditions of hypoglycemia. It would appear then that this supply may be quickly replenished by a temporary rise in blood sugar. Another explanation may involve acetylcholine synthesis which is said to be increased in nervous tissues during hypoglycemia.[13] Finally, energy requirements of nervous tissue in ruminants may be met at least partially by oxidation of substances other than glucose. Reid's[16] observation of a significant A-V* difference in acetate in the sheep's head may suggest a difference between ruminant and nonruminant neural metabolism. Whatever the explanation, recovery following a short period of high blood glucose was progressive and obvious during subsequent hypoglycemia.

This series of experiments also indicate, as did the work of Reid[17] in sheep, a marked resistance to critical levels or durations of hypoglycemia. Study of the curves indicates a somewhat irregular response to insulin and a definite tendency for the blood glucose to increase in spite of high levels of insulin.

The tendency of the blood glucose to reach a steady state at a minimum of about 5 mg. per 100 cc. under maximum insulin influence was not as obvious as in the case of sheep observed by Reid.[17] A more marked difference was the lack of evidence that 5 mg. per 100 cc. or any other level was critical in the sense that maintenance at this or a lower level was essential to the development of marked symptoms.

SUMMARY AND CONCLUSIONS

1) Two units of insulin per kilogram intravenously gave a maximum effect in reducing blood glucose, whereas 1 unit did not.

2) Increasing insulin dosage up to 10 units per kilogram increased duration but not depth of hypoglycemia.

3) The blood sugar level, even under the influence of large insulin injections, is variable, indicating a vigorous resistance to very low glucose levels.

4) The rate of fall of blood glucose after insulin injection was relatively constant, appearing to be between 25 and 40 mg. per 100 cc. of blood in thirty minutes. The rate was independent of insulin dosage.

5) Hypoglycemic response to intravenous insulin may be almost immediate or delayed for over thirty minutes postinjection.

6) Mild hypoglycemic symptoms were observed in 1 of 2 cows receiving single injections of 2, 5, and 10 units of insulin per kilogram.

7) Prolonged insulin hypoglycemia will result in a convulsive, hypoglycemic crisis, which responds well to glucose injections.

8) Recovery from hypoglycemic symptoms was observed to follow a short period of high blood glucose despite a long con-

* Arteriovenous.

tinued hypoglycemia immediately thereafter.

9) Cows are able to withstand short periods of extreme hypoglycemia much more readily than a prolonged period at a significantly higher blood glucose level.

References

[1] Amadon, R. S.: Experimental Study of Milk Fever. Vet. Extension Quart., *29*, (1928): 1-2.

[2] Auger, L.: Sur la Theorie de L'Hypoglycemia dans. la Fievre Vitulaire. Rev. Gen. de. Méd. Vét., *35*, (1927): 353-384. Cornell Vet., *17*, (1927): 64-73.

[3] Bodansky, A.: Antagonistic Effects of Insulin and Thyroxin. Proc. Soc. Exptl. Biol., *20*, (1923): 538-540.

[4] Bodansky, A.: Effects of Dosage and Previous Diet on Blood Sugar Curves in Sheep after Intravenous Administration of Insulin. Proc. Soc. Exptl. Biol. Med., *21*, (1924): 416-417.

[5] Cutler, J. T.: Studies on the Carbohydrate Metabolism of the Goat. The Blood Sugar and the Inorganic Phosphate. J. Biol. Chem., *106*, (1934): 656-666.

[6] Dworkin, S.: The Response of Sympathectomized Animals to Insulin. Am. J. Physiol., *98*, (1931): 467-474.

[7] Fisher, Peter: The Role of Ketone Bodies in the Etiology of Diabetic Coma. Am. J. M. Sci., *221*, (1951): 384-397.

[8] Folin, O., and Malmros, H.: An Improved Form of Folin's Micromethod for Blood Sugar Determinations. J. Biol. Chem., *83*, (1929): 115-121.

[9] Himwich, H. E., Bowmen, K. M., Wortis, J., and Fazekos, J. F.: Metabolism of the Brain During Insulin and Metrazol Treatments of Schizophrenia. J. Am. M. A., *112*, (1939): 1572.

[10] Hitchcock, M. W. S., and Phillipson, A. J.: The Tolerance of Sheep to Low Concentrations of Blood Sugar. J. Physiol., *105*, (1946): 42.

[11] Hupka, E.: Die Azetonemie der Rinder. Deutsche tierärztl. Wchnschr., *36*, (1928): 98-101.

[12] Jasper, Donald E.: Hepatic Changes in the Bovine During Pregnancy and Ketosis. Doctor of Philosophy Thesis, University of Minnesota, St. Paul, 1947.

[13] Mason, M. F., and Emerson, Kendall, Jr.: Principal Pathways of Intermediate Metabolism. Harrison, T. R., ed., Principles of Internal Medicine, 1st ed. The Blakiston Co., Philadelphia, Pennsylvania, 1950.

[14] Nelson, Norton: A Photometric Adaption of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., *153*, (1944): 375-380.

[15] Petersen, W. E., Hewitt, E. A., Boyd, W. L., and Brown, W. R.: Artificially Induced Hypoglycemia and Hypocalcemia in the Cow and the Relationship to Parturient Paresis or Milk Fever. J.A.V.M.A., *79*, (1931): 217-227.

[16] Reid, R. L.: Studies on the Carbohydrate Metabolism of Sheep. II. The Uptake by the Tissues of Glucose. Austral. J. Agric. Res., *1*, (1950): 338-354.

[17] Reid, R. L.: Studies on Carbohydrate Metabolism of Sheep. III. The Blood Glucose During Insulin Hypoglycaemia. Austral. J. Agric. Res., *2*, (1951): 132-145.

[18] Reid, R. L.: Studies on Carbohydrate Metabolism of Sheep. IV. Hypoglycemic Signs and Their Relation to Blood Glucose. Austral. J. Agric. Res., *2*, (1951): 146-157.

[19] Roepke, M. H.: Physiologic and Metabolic Aspects of Acetonemia in Cattle. J.A.V.M.A., *100*, (1942): 411-414.

[20] Sampson, J., and Boley, L. E.: The Physiologic Basis of Therapy for Ketosis in the Ruminant. Am. J. Vet. Res., *2*, (1941): 327-332.

[21] Sampson, Jesse, and Hayden, C. E.: The Acid-Base Balance in Cows and Ewes During and After Pregnancy, with Special Reference to Milk Fever and Acetonemia. J.A.V.M.A., *86*, (1935): 13-23.

[22] Schunecker, Karl: Beobachtungen uber den Blutzucker der Ziege. Biochem. Ztschr., *156*, (1925): 353-364.

[23] Shaw, J. C.: Studies on Ketosis in Dairy Cattle. VIII. The Efficiency of B Vitamins and Methionine in the Treatment of Ketosis. J. Dai. Sci., *29*, (1946): 131-139.

[24] Shaw, J. C.: Studies on Ketosis in Dairy Cattle. VIII. Spontaneous Recovery. J. Dai. Sci., *29*, (1946): 151-155.

[25] Shaw, J. C., Matterson, L. D., Surgenor, M. E., and Hourigan, C. A.: Studies on Ketosis in Dairy Cattle. VI. Is Ketosis in Dairy Cattle Due to a Vitamin A Deficiency? J.A.V.M.A., *106*, (1945): 285-291.

[26] Strand, R., Anderson, W., and Allcroft, W. M.: Further Studies on the Lactic Acid, Sugar, and Phosphorus of the Blood of Ruminants; (a) Following Adrenalectomy and (b) After Intravenous Injection of Insulin. Biochem J., *28*, (1934): 642-649.

[27] Widmark, E. M. P., and Carlens, Olaf: Uber die Blutzuckerkonzentration bie Kuhen und den Einfluss der Lactations Intensitett auf dieselbe. Biochem. Ztschr., *156*, (1925): 454-459.

[28] Zuker, T. F., and Berg, B. N.: The Role of the Adrenal Gland in Blood Sugar Recovery After Insulin Hypoglycemia. Am. J. Physiol., *119*, (1937): 539-548.

Studies on a Nonspecific Brucella-Agglutinating Substance in Bovine Serum. I. The Differentiation of the Specific and Nonspecific Agglutinins by Heat Treatment

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BRUCELLA agglutination reactions in the suspicious titer range (1 : 50-1 : 100) are encountered in serums from some animals from apparently noninfected herds. A number of such serums were tested with a variety of bacterial antigens in an endeavor to demonstrate cross reactions which could account for the Brucella agglutination titers. Among the organisms tested were a strain of Proteus 0X19, *Salmonella typhosa, Salmonella gallinarum, Salmonella pullorum, Salmonella typhimurium, Alcaligenes fecalis,* and a species of Micrococcus. Many of the serums agglutinated all of the above mentioned antigens as well as *Brucella abortus.* The titers were uniformly low, and absorption studies failed to disclose any definite cross reactions. In fact, the agglutinating activities of three of the serums appeared to be completely lacking in specificity, and any one of the antigens tested could be used to remove the agglutinins for all of the others, including Brucella. With certain other serums which agglutinated all the antigens mentioned, it was found that absorption with Brucella removed all the various agglutinins. On the other hand, exhaustive absorption with any one of the other antigens resulted in the removal of agglutinins for all the antigens except Brucella. The Brucella titer was lowered only slightly by this procedure. Thus, specific and nonspecific Brucella agglutinins were in evidence. Because of their diversity of action, it was believed that the nonspecific agglutinins might be substances differing quite markedly from antibodies of the classical type, and the possibility of distinguishing the two types of agglutinating substances by physicochemical means presented itself.

It was known that antibodies in serum could be destroyed or inactivated at certain temperatures. Also, it was shown that these temperatures were not the same for all antibodies.[1,2,3] Heat treatment was tested as a possible means of differentiating the specific and nonspecific Brucella agglutinins in bovine serums. Preliminary tests were carried out on serums having well-defined Brucella-agglutinating activity of one type or the other. Specific serums were first absorbed with Proteus 0X19 to assure the removal of any nonspecific agglutinins. The three serums which had previously been found to display a broad range of agglutinating activities and no apparent specificity were tested as nonspecific serums. It was soon found that a heat treatment of 70 C. for ten minutes served to inactivate the nonspecific agglutinins. The specific serums were able to withstand 70 C. for an hour with no measurable drop in titer. At 90 C. for ten minutes, inactivation of the specific agglutinins for Brucella became apparent. The aforementioned heat treatments could be successfully applied only if the serums were first diluted with equal parts of saline in order to prevent coagulation.

In a concurrent study, the results of which have been presented elsewhere,[4] a filter paper chromatographic method was developed which made possible the differentiation of the specific and certain nonspecific Brucella-agglutinating substances in serum on the basis of their affinity for adsorption on cellulose. The specific agglutinins migrated with the solvent on the paper, while the nonspecific agglutinating material remained adsorbed at the point where the serum was applied to the paper. This test, along with the heat treatment, was applied to a collection of Brucella-agglutinating bovine serums. The present report deals with the problems encountered and the results obtained in this study

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and is offered especially to point out some of the differential properties which served to characterize the two types of agglutinins.

MATERIALS AND METHODS

Brucella-agglutinating bovine serums were obtained from the brucellosis testing laboratories of the U. S. Bureau of Animal Industry at the University of Minnesota. They were placed in three groups on the basis of the herds of origin. Nine were serums of suspicious titer from animals in suspect herds. Fifteen serums were from suspect animals in herds containing reactors. Another fifteen serums were from reactors. Each serum was diluted 1 : 2 with 0.85 per cent saline. A 1-ml. portion was immersed for ten minutes in a water bath held at 70 C. Heated and unheated samples of each serum were then serially diluted with 0.85 per cent saline. To a 1-ml. volume of each dilution, 1 ml. of double strength U. S. Bureau of Animal Industry's *Br. abortus* tube antigen was added. The tests were read after incubation at 37 C. for forty-eight hours.

When this procedure was applied to the serums in this study, it was found that a few of the serums having high titers manifested the prozone phenomenon after heat treatment. The prozone in some cases extended beyond the limits of the average nonspecific titer (1 : 100). In experiments with serums showing prozones, Jones and Orcutt [5] demonstrated that the combination of antibody with the bacterial cell had taken place in the prozone dilutions; however, agglutination was inhibited by the presence of a substance which reduced the cohesive forces between the sensitized antigen particles. The material could be removed by centrifugation of the tubes displaying the prozone. When the sedimented cells were resuspended in saline, they immediately agglutinated. If a heat treatment of certain serums of high titer could produce substances capable of blocking agglutination in dilutions as high as 1 : 100, it was believed that the same might occur in certain serums of suspicious titer. If such were the case, specific agglutination in this type of serum would not be seen, and the results might be misinterpreted as indicating complete inactivation of the agglutinins. Therefore, after the first reading of the tests, the tubes containing the reaction mixtures were centrifuged at 3,000 r.p.m. for thirty minutes. The supernatant liquid was discarded and the original volume was restored with fresh 0.85 per cent saline. The antigen was resuspended by vigorous shaking. After standing an hour at room temperature, the tests were again read. The unheated samples treated in the same way served as controls. Undiluted samples of each serum were tested by the filter paper chromatographic method as described in a previous report.[4]

RESULTS

The three series of serums tested were identified by the prefixes "SS," "SR," and "R." SS denoted the serums of suspicious titer from animals in suspect herds,

TABLE 1—Summary of Agglutination Reactions of 39 Bovine Serums Before and After Heat Treatment

Reaction type	No. serums classified as: SS*	SR**	R†	Serum (No.)	Heat treatment‡	Resuspension §	Examples of reaction types: Brucella agglutination in dilutions indicated: x	10	16	20	32	40	64	80	128	160	256	320	512
							1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :	1 :
Type 1. (no change)	1	4	9	R3	none	before	+	+	+	+	+	+	+	+	+	+	—	—	
					none	after	+	+	+	+	+	+	+	+	+	+	—	—	
					treated	before	+	+	+	+	+	+	+	+	+	I	—	—	
					treated	after	+	+	+	+	+	+	+	+	+	+	—	—	
Type 2. (prozone)	0	0	6	R8	none	before	+	+	+	+	+	+	+	+	+	+	+	—	
					none	after	+	+	+	+	+	+	+	+	+	+	+	—	
					treated	before	—	—	—	—	—	—	—	—	+	+	—	—	
					treated	after	+	+	+	+	+	+	+	+	+	+	—	—	
Type 3. (masked)	2	9	0	SR13	none	before	+	+	+	+	+	+	—	—	—	—	—	—	
					none	after	+	+	+	+	+	+	—	—	—	—	—	—	
					treated	before	—	—	—	—	—	—	—	—	—	—	—	—	
					treated	after	+	+	I	—	—	—	—	—	—	—	—	—	
Type 4. (inactivated)	6	2	0	SS12	none	before	+	+	+	+	+	+	I	—	—	—	—	—	
					none	after	+	+	+	+	+	+	I	—	—	—	—	—	
					treated	before	—	—	—	—	—	—	—	—	—	—	—	—	
					treated	after	—	—	—	—	—	—	—	—	—	—	—	—	

* SS = suspect serum from animal in suspect herd; ** SR = suspect serum from animal in reactor herd; † R = reactor serum; ‡ = 70 C. for 10 minutes; § antigen in each tube was sedimented by centrifugation and resuspended in 2 ml. of saline.

+ = complete agglutination; — = no agglutination; I = incomplete agglutination.

while SR identified the serums of suspicious titer from herds containing reactors. The R series was made up of serums of infected or reactor animals. Each of the 39 serums tested was found to fit one or another of four general types of reactions. These reaction types were characterized as follows:

Type 1 (No Change).—The heated and unheated samples had the same titer. The filter paper chromatographic reaction was specific.

Type 2 (Prozone).—The heated samples showed a reduction in titer and a prozone. After centrifugation and resuspension, the prozone was abolished while the reduced titer persisted. Reactions of this type were only seen in serums having titers of 1 : 200 or higher in the unheated samples. The filter paper test disclosed the presence of both specific and nonspecific agglutinins.

Type 3 (Masked).—Agglutinating activity was apparent in the heated samples only after the antigen was resuspended in fresh saline. This type of reaction was believed to be essentially the same as type 2 with the exception that it occurred only in serums having a titer of 1 : 100 or less. The material capable of blocking agglutination was apparently present in a concentration sufficient to completely mask the activity of the heat-stable agglutinins of low titer. Here again the mixed reaction was given on the chromatogram.

Type 4 (Inactivated).—In serums of this type, the agglutinating activity was completely destroyed by the heat treatment and could not be restored by centrifugation and resuspension of the antigen. The chromatographic reaction in this case was nonspecific only.

The results of this study are summarized in table 1. The number of serums of each series fitting each of the general types of reactions is tabulated; also, reactions obtained with representative serums are presented as illustrations of the general types. Six of the nine serums of the SS series no longer agglutinated *Br. abortus* after the heat treatment. From these results, it appears that about two thirds of the suspicious reactions encountered in herds that are free of reactors are due to nonspecific agglutinins. This ratio has been substantiated on a larger number of serums by the filter paper chromatographic method.[4]

DISCUSSION

The data indicated that serums, in which the agglutinin-blocking materials were present after heat treatment, had contained both specific and nonspecific agglutinating substances. It appeared that the prozones or the complete inhibition of agglutination in certain serums of low titer might have been caused by the presence of inactivated nonspecific substances. Subsequent studies with purified preparations of the nonspecific agglutinating material indicated that the substance was much more resistant to inactivation by heat after it had been largely freed of other serum constituents. It may be that its inactivation in serum by heating at 70 C. for ten minutes was due to the formation of a complex with some other serum constituent rather than to destruction or denaturation by heat. Regardless of the mechanism of the loss of agglutinating activity on heating, this property along with the strong affinity for adsorption on cellulose and the lack of specificity in agglutinating activity was taken to indicate that the nonspecific substances were not antibodies of the classical type. This belief has been fortified by information that has come out of studies on the purification of the two types of agglutinating substances. These studies are the subject of another report.

SUMMARY

A nonspecific Brucella-agglutinating substance in bovine serum has been differentiated from the specific Brucella agglutinin on the basis of its lack of specificity in agglutinating activity, its strong affinity for adsorption on cellulose, and its inactivation by heat treatment of 70 C. for ten minutes.

References

[1] Beyer, H. G., and Reagh, A. L.: The Further Differentiation of Flagellar and Somatic Agglutinins. J. M. Res., *12*, (1904): 313-328.

[2] Jones, F. S.: The Effect of Heat on Antibodies. J. Exptl. Med., *46*, (1927): 291-301.

[3] Kleczkowski, A.: Effect of Heat on Flocculating Antibodies of Rabbit Antisera. Brit. J. Expti. Path., *22*, (1941): 192-208.

[4] Hess, W. R., and Roepke, M. H.: A Nonspecific Brucella Agglutinating Substance in Bovine Serum. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 469-472.

[5] Jones, F. S., and Orcutt, M.: The Prozone Phenomenon in Specific Bacterial Agglutination. J. Immunol., *27*, (1934): 215-233.

Studies on a Nonspecific Brucella-Agglutinating Substance in Bovine Serum. II. Isolation and Purification of the Brucella-Agglutinating Substances

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THE PRESENCE of nonspecific Brucella-agglutinating substances in bovine serums has been demonstrated.[1,2] A lack of specificity in agglutinating activity, a lower resistance to inactivation by heat, and an affinity for adsorption on cellulose were the properties which served to distinguish these substances from the specific Brucella agglutinins in serum. To make further characterization possible, the isolation and purification of the agglutinating substances were undertaken. It is the purpose of this report to describe the methods used and point out additional differential characteristics observed during the isolation and purification processes.

MATERIALS AND METHODS

The Brucella-agglutinating serums studied were from 3 cows. Two were suspect animals from herds that were otherwise free from reactors. Both animals had been classed as suspicious on each of several retests conducted over a period of several months. Their serums consistently gave nonspecific reactions on the filter paper chromatogram. These serums were designated by the numbers 846 and 883. The third animal was a condemned Brucella reactor. Serum from this animal obtained at the time of slaughter had a titer of 1 : 800. Its designation was P40.

Adsorption on Cellulose.—Of the various cellulose materials that were found to serve as adsorbents for the nonspecific substances, solka floc bw 200* was used extensively because it was particularly suited to the preparation of uniform adsorption columns. A serum diluted with an equal volume of a 0.05 M phthalate solution at pH 6.2 was allowed to seep through a column of the adsorbent. The column was washed free of unadsorbed serum constituents by passing a large volume of 0.9 per cent saline through it. The adsorbed agglutinating material was eluted by washing the column with distilled water. The eluate was collected in a number of small tubes. A 0.08-ml. sample from each tube was tested by the rapid plate method for Brucella-agglutinating activity. The contents of the tubes displaying activity were pooled and further concentration of the agglutinating substance was achieved by ammonium sulfate precipitation. The ammonium sulfate was removed by dialysis.

Adsorption on Brucella Abortus Antigen.—One or 2 liters of serum were placed in a separatory funnel and thoroughly mixed with a heavy suspension of *Brucella abortus* antigen. The mixture was allowed to stand at room temperature overnight. On the following day, the agglutinated antigen along with a small amount of serum was drawn from the bottom of the funnel and distributed in centrifuge tubes. The antigen was sedimented by centrifugation, resuspended in saline, and again spun down. After three such washings with saline, the antigen was resuspended in double-distilled water and centrifuged. Four washings of this type were made, and the supernatant liquids were pooled. The agglutinating substances were recovered from the washings by precipitation with ammonium sulfate. The ammonium sulfate was removed by dialysis.

Isolation of Agglutinating Substances by Serum Fractionation.—Method 10 of Cohn, *et al.*[3] was the procedure followed in this study. Separation of the serum components was achieved by precipitation and extraction under controlled conditions of pH, ionic strength, protein concentration, temperature, and alcohol concentration. The filter paper chromatographic method was used to detect the fractions possessing agglutinating activity.

RESULTS

The agglutinating substances present in the nonspecific reacting serums 846 and 883 were adsorbed on the cellulose column. The substances were colorless but could be located by washing a hematoxylin-stained Brucella antigen through the column. The antigen became fixed in the region where the agglutinating substances were adsorbed. The region formed a broad, rather diffuse band near the top of the column. There was no adsorption of the specific Brucella agglutinins of serum P40. Of the various eluting agents tested, distilled wa-

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ter gave the best results. Yet, as indicated by the volumes and titers of the serums and eluates, it was never possible to recover more than 35 per cent of the adsorbed agglutinating substances from the columns. If after elution with distilled water, the adsorbent was washed with 0.1 N sodium hydroxide, additional material was eluted. This material had no agglutinating activity. It rapidly settled out upon standing and could not be redissolved by neutralizing or moderately acidifying the solution. The substance was apparently denatured protein, but whether it was protein that had been altered by adsorption on cellulose or by treatment with alkali could not be determined, for it was never possible to elute it by less drastic means.

TABLE 1—Recovery of Brucella-Agglutinating Substances from Bovine Serums by Adsorption on and Elution from Brucella Organisms

Serum (No.)	Serum titer	Serum volume (ml.)	Eluate titer	Eluate volume (ml.)	Yield of** agglutinating substance (%)
846	1 : 50	1,000	1 : 1,600	12	38.4
883	1 : 75*	1,000	1 : 1,600	16	34.1
P40	1 : 800	100	1 : 256	5	1.4

* Partial agglutination in the 1 : 100 dilution.

** Sample calculation of yield:

1,000 ml. of serum having titer of 1 : 50 = 50,000 agglutinating units

12 ml. of eluate having titer of 1 : 1,600 = 19,200 units

$$\frac{19{,}200}{50{,}000} \times 100 = 38.4\ \%$$

Brucella antigen was tried as an adsorbent in hopes that the process might have a greater degree of reversability than the adsorption on cellulose. The results obtained are presented in table 1. Both the specific and nonspecific agglutinating substances were, of course, adsorbed from the serums by this method, but there was a marked difference in the ease with which the two substances could be removed from the organisms. When the agglutinins were precipitated from the eluates by treatment with ammonium sulfate, another difference between the specific and nonspecific materials was evidenced. The nonspecific agglutinating substance was completely precipitated at 30 per cent saturation, while up to 50 per cent saturation with ammonium sulfate was required for the removal of the specific agglutinin. Further differential characteristics were revealed by the fractionation of serum. The specific agglutinins were found to be associated with the *gamma* globulin fraction of the serum, while the nonspecific agglutinating substances were associated with a fraction which Cohn has designated as containing isoagglutinins, cold insoluble globulins, and certain constituents concerned with the clotting of blood. A separation of the components of the last mentioned fraction was not achieved.

DISCUSSION

Although the sharing of similar antigens by certain organisms is a well-known cause of nonspecific serological reactions, evidence has been presented which indicates that this mechanism is not the most important cause of the low titered Brucella agglutination reactions often encountered with serums of healthy cattle from apparently noninfected herds. Instead, the agglutinating activity of approximately 60 per cent of these serums is due to substances which differ in several respects from agglutinins of the classical type.[1] The bases upon which these substances have been differentiated from the specific Brucella agglutinins are summarized in table 2.

TABLE 2—Differences Between the Specific and Nonspecific Brucella-Agglutinating Substances in Bovine Serums

Bases for the differentiation of agglutinating substances	Nonspecific substance	Specific agglutinin
Removal from serum by adsorption with bacteria	Accomplished with a variety of organisms	Accomplished with Brucella only
Heat treatment required for inactivation	70 C. for 10 minutes	90 C. for 10 minutes
Adsorption on cellulose	Adsorbed	No adsorption
Yield obtained by elution from *Brucella abortus* with distilled water	Up to 38 %	Less than 2 %
Concentration of ammonium sulfate required for precipitation	30 % saturation	50 % saturation
Relationship to the *gamma* globulins	No association	Contained in the *gamma* globulin fraction

Demonstrable amounts of nonspecific substances are present in the serums of many apparently healthy animals of a variety of species. The origin and biological importance of these materials are unknown. It seems likely that they are normal serum constituents and probably are the same as those substances which have been designated as natural antibodies by Burgi,[4] Gibson,[5] and others.

SUMMARY

During the course of isolating and purifying certain nonspecific Brucella-agglutinating substances in bovine serum, additional properties which serve to distinguish these substances from the specific Brucella agglutinins have been demonstrated.

1) Cellulose adsorption columns were used to remove the nonspecific substance from serum. The specific Brucella agglutinins were not adsorbed on cellulose.

2) Nonspecific substances adsorbed on Brucella organisms were more readily eluted than were the specific agglutinins.

3) The nonspecific materials were precipitated from eluates by 30 per cent saturation with ammonium sulfate, while up to 50 per cent saturation was required for the precipitation of the specific agglutinins.

4) Specific Brucella agglutinins were found to be associated with the *gamma* globulin fraction of the serum. The nonspecific substances were associated with a serum fraction containing isoagglutinins, cold insoluble globulins, and certain factors concerned with the clotting of blood.

References

[1] Hess, W. R., and Roepke, M. H.: A Nonspecific Brucella Agglutinating Substance in Bovine Serum. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 469-472.

[2] Hess, W. R.: Studies on a Nonspecific Brucella Agglutinating Substance in Bovine Serum. I. The Differentiation of the Specific and Nonspecific Agglutinins by Heat Treatment. Am. J. Vet. Res., *14*, (1935): 192-194.

[3] Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahut, F. W., Leever, W. F., Liu, C. H., Mittleman, D., Mouton, R. F., Schmid, K., and Uroma, E.: A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma. J. Am. Chem. Soc., *72*, (1950): 465-474.

[4] Bürgi, E.: Uber Bakterienagglutination durch Normale Sera. Arch. f. Hyg., *62*, (1907): 239-276.

[5] Gibson, H. J.: Observations on the Occurrence, Characteristics, and Specificity of Natural Agglutinins. J. Hyg., *30*, (1930): 337-356.

Experimental Infections of Calves with the Nematode, *Nematodirus Helvetianus*

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NEMATODIRUS HELVETIANUS is a trichostrongylid nematode of the small intestine of cattle and, occasionally, sheep, goats, and camels. According to Dikmans,[1] the first report of *N. helvetianus* in the United States was in 1928 when E. W. Price, of the Bureau of Animal Industry, identified specimens collected from cattle in Cheyenne, Wyo. Since that time, Stoll,[2] Porter,[3] Cooperrider,[4] Krohn,[5] and others have reported the occurrence of this parasite in different sections of this country.

Commonly known as the thread-necked strongyle, *N. helvetianus* is generally regarded as relatively nonpathogenic to cattle. However, Tunnicliff[6] reported parasitism in a herd of 500 short yearlings on range, in which 15 were noticeably sick; of the latter, the 2 most seriously affected died. The feces showed few coccidia and Nematodirus eggs in 1 calf; many coccidia and massive infection of strongyles were revealed upon postmortem examination of the second calf. The worms recovered from the duodenum and abomasum were identified as *Cooperia oncophora* and *N. helvetianus*. The only evident antemortem symptoms were emaciation, roughened coat, dull sunken eyes, and a thin, profuse, more or less bloody diarrhea. Tunnicliff questioned whether the coccidia or the nematodes were the primary cause of death.

Curtice,[7] Ryksen,[8] and others have long maintained that Nematodirus spp. were serious pests in sheep. More recently, Kates and Turner[9] observed that lambs experimentally infected with large numbers of larvae of *Nematodirus spathiger* exhibited symptoms of parasitism, including diarrhea, loss of appetite, and general weakness and emaciation. Also, infected lambs made poorer weight gains than the controls. Since *N. helvetianus* is rather widely distributed in the United States and in other parts of the world, and is usually found as part of mixed nematode infections in calves, the authors of this paper considered it desirable to determine the specific effects of this parasite on the health of calves. The results of the experiments we performed are the subject of this report.

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Materials and Methods

The animals used were grade Jersey calves, obtained from local dairies, within twenty-four hours after birth. These calves were raised free of helminths in portable pens[10] until they were from 4 to 5 months old, when they were transferred to individual stalls in concrete-floored barns. Droppings were removed and the stall floors were washed daily. Infected and control calves were given identical rations of alfalfa hay and grain. Milk was fed until the calves were 4½ months old.

Larvae were cultured from eggs recovered from the feces of a calf with a natural infection of *N. helvetianus*. These larvae were used for the initial infection. Eggs were subsequently recovered from the feces using a technique of sedimentation, followed by centrifugal flotation in concentrated salt solution. Eggs thus recovered were washed and cultured in water in shallow Petri dishes at 28 C.

The numbers of larvae fed to calves were determined by dilution count in a Scott counting chamber. The infective larvae were administered orally in a few cubic centimeters of water through a dissecting blow pipe attached to a 5-cc. rubber bulb.

The course of infection was followed by fecal egg counts, using the Stoll[11] technique with $ZnSO_4$ (sp. gr. 1.18) as the flotation solution. All calves were checked routinely by the Wintrobe hematocrit method for packed red cell counts. Weights were recorded weekly.

Observations and Data

The first animal infected experimentally was a 5-month-old calf (1122) which was fed 620 larvae in one dose. The calf passed eggs on the twenty-first day of the infection period, and continued to pass them for 115 days; during this time the fecal count never exceeded 8 e.p.g.* There was no evidence of anemia or other symptoms of parasitism. During the thirteen-week observation period the infected calf gained 2 lb. more than a comparable control (1120) this being, of course, purely accidental.

In a second experiment, a 9-month-old calf (1150) was fed 27,000 larvae in ten days, the number of larvae given per day ranging from 700 to

* Eggs per gram of feces.

5,000. Eggs were passed twenty-one days after the initial dose. The egg count reached a peak of 196 e.p.g. in fourteen days and became negative forty-six days after the first recovery of eggs. Hematocrit readings remained at a normal level. To all outward appearances, the calf was unaffected by its parasite load. However, definite retardation in the rate of gain was evident as early as two weeks after infection (table 1). This retardation was still apparent six weeks after infection, the control (1170) having gained 22 lb. more than the infected animal.

TABLE I—Weight Gained by Animals Infected with Nematodirus and by Their Controls

Calf No.	Gain in pounds at various periods of infection			
	2 weeks	6 weeks	13 weeks	24 weeks
1122 (1120)	10 (3)	30 (31)	55 (53)	.....
1150 (1170)	8 (18)	18 (40)	.....	.....
1187 (1190)	2 (10)	27 (65)	69 (92)	.....
1202 (1201)	—5 (9)	15 (25)	45 (75)	97 (130)
1203 (1201)	—5 (9)	5 (25)	50 (75)	100 (130)

Figures in parenthesis are for uninfected controls.

In a third experiment, a 7-month-old calf (1187) passed eggs twenty-four days after having been fed 140,000 larvae in daily doses of 32,000, 75,000, and 33,000 over a three-day period. The calf passed eggs for sixty-six days, and reached a peak of 120 e.p.g. on the forty-fourth day. As in the previous experiment, calf 1187 appeared perfectly normal, but the rate of gain was adversely affected. Table 1 shows that the infected calf gained only 2 lb. during the first two weeks of the infection period, whereas a comparable control gained 10 lb. in this period. Six weeks later the infected calf had gained 27 lb., which was 38 lb. less than that of the control. Thirteen weeks after initiating the experiment, the setback in weight was still apparent, as the control had gained 33 per cent more weight than the infected calf.

In a fourth experiment, 2 calves, 5½ months old, were given a million larvae each, calf 1203 in one dose, and calf 1202 in four equal doses, in as many days. Calf 1203 passed eggs after twenty-one days and continued to do so for the next twenty-eight days. The peak was never higher than 14 e.p.g. No eggs were found in the feces of calf 1202. Table 1 shows that the infected animals each lost 5 lb. during the first two weeks of the infection period, whereas an uninfected calf of the same age (1201) gained 9 lb. during this period. Six weeks after infection, the infected calves had gained 40 and 80 per cent less weight, respectively, than the control; after thirteen weeks the difference was 40 and 33 per cent, respectively, showing considerable recovery. When these 2 animals were transferred to other experiments six months later, the setback was still in evidence, though it had become somewhat reduced.

Both infected calves began to scour fifteen days after the initial dose of infective larvae, calf 1202 scouring for fourteen days, and calf 1203 for eleven days. The diarrhea was thin and profuse but not bloody. Three days after the onset of diarrhea, the calves showed anorexia and did not feed normally for five days. Although no anemia was noted, both infected animals became weak and emaciated, lying on the floors of their stalls and rising sluggishly. The uninfected control was normal throughout the entire period of observation.

Discussion

The data indicate that when calves were exposed to large numbers of infective larvae of the thread-necked strongyle, *N. helvetianus*, definite pathogenic effects were produced, which varied in intensity with the number of larvae ingested. When only a few hundred larvae were administered to a calf, there were no signs of detrimental effects on the health and growth of the animal. In 2 calves to which several thousand larvae were administered, no clinical symptoms of parasitism were noted, but there was definite retardation in the rate of growth. The infected animals continued to gain weight but at a slower rate than the uninfected controls. In the two cases involving massive numbers of larvae, clinical symptoms of parasitism were clearly evident, including diarrhea, anorexia, and general weakness, in addition to retarded growth. The setback in weight gains were evident for as long as six months after the infection.

Two weeks after infection, marked retardation of growth was evident in 4 calves (1150, 1187, 1202, and 1203), and an actual loss of weight had been sustained by 2 (1202 and 1203) of the animals. During this time, the parasites were developing. The appearance of clinical symptoms in calves 1202 and 1203, on the fifteenth day after infection, coincided with the period during which these parasites developed from young adults to sexually mature worms, as shown by the recovery of unensheathed worms, about 6 mm. long, from a calf killed fifteen days after having been fed larvae.* Therefore, the evidence from the life history of the worms shows that the prepatent or developmental period of the parasite corresponds to the acute stage of infection. Similar observations were recorded in connection with studies of *Haemonchus contortus* and *Oesophagostomum radiatum* by Mayhew.[12,13]

* Unpublished data.

While following the development of this helminthic infection by fecal eggs counts, it was noted that the maximum output of eggs, namely, 196 and 120 e.p.g., was obtained from calves 1150 and 1187, respectively, which showed no clinical symptoms after having been fed several thousand larvae. Contrariwise, the 2 calves showing symptoms of parasitism after having been fed 1 million larvae each passed only a maximum of 14 e.p.g. in 1 case and none in the other calf. Mayhew [14] reported similar results in experimental infections of calves with the hookworm, *Bunostomum phlebotomum*. Unfortunately, neither of our calves fed massive numbers of larvae was killed for postmortem examination. Consequently, it is impossible to determine whether the poor egg production resulted from a crowding of the adult nematodes, with a subsequent suppression of egg laying, or whether few of the larvae in these massive doses were able to develop into adults. Regardless of which condition prevailed, the result affords further evidence of the fact that diagnosis of parasitism, especially the extent of infection, can not be based on egg counts only but must necessarily include observation of symptoms, rate of growth and, if possible, postmortem examination of a typically affected animal in the herd.

The experiments indicate, moreover, that *N. helvetianus* is potentially pathogenic to calves and, if occurring in sufficient numbers, contributes to the ill effects of the aggregate parasitism acquired by susceptible calves when grazing on contaminated pastures.

—*Leonard Reid Davis*

Fig. 1—Adult Nematodirus helvetianus. 1—female; 2—male.

SUMMARY

1) Experimental infections with the thread-necked strongyle, *Nematodirus helvetianus*, were established in 5 calves, 5 to 9 months old, by feeding the infective larvae.

2) One 5-month-old calf given a few hundred larvae was unaffected. Two other calves fed 27,000 and 140,000 larvae, respectively, had gained 55 per cent and 59 per cent less weight than comparable controls six weeks after infection; neither of these calves showed symptoms of parasitism.

3) Two calves fed 1 million infective larvae each also showed retarded weight gain, a control calf gaining 40 to 80 per cent more than those infected, in six weeks. Six months from the date of infection, the setback in weight was still apparent, though not as pronounced. During the third and fourth weeks of infection, the calves exhibited symptoms of diarrhea, loss of appetite, and weakness.

4) *Nematodirus helvetianus* exerted its most detrimental effect on calves during the prepatent period, as shown by the fact that fourteen days after infection there was a marked retardation of weight gain in 4 infected calves; in 2 of these animals, clinical symptoms appeared at this same time.

References

[1] Dikmans, G.: *Nematodirus helvetianus* in the U. S. J. Parasitol., *18*, (1931): 56.

[2] Stoll, N. R.: Observations on Cattle Nematode Infections, with a Demonstration of their Secondary Transmission to Sheep. J. Parasitol., *22*, (1936): 386-407.

[3] Porter, D. A.: Incidence of Gastrointestinal Nematodes of Cattle in the Southeastern United States. Am. J. Vet. Res., *3*, (1942): 304-308.

[4] Cooperrider, D. E., Pearson, C. C., and Kliewer, I. O.: A Survey of the Gastrointestinal Parasites of Cattle in Oklahoma. Oklahoma Agric. Exper. Sta. Tech. Bull. No. T-31, (1948): 18.

[6] Krohn, A. F.: New Record of a Nematode in Michigan. M.S.C. Vet., *10*, (1950): 129.

[7] Tunnicliff, E. A.: The Occurrence of *Cooperia oncophora* and *Nematodirus helvetianus* in Calves. J. A. V. M. A., *80*, (1932): 250-251.

[8] Curtice, C.: Nematodirus in Sheep a Serious Pest. J. Parasitol., *9*, (1922): 39.

[9] Ryksen, W. J.: The Occurrence and Pathogenicity of Nematodirus Species in Arid Areas. J. South African Vet. M. A., *10*, (1939): 29-31.

[10] Kates, K. C., and Turner, J. H.: Observations on the Pathogenicity of *Nematodirus spathiger* in Lambs. J. Parasitol., *35*, (1949): 13.

[11] Davis, L. R.: Portable Pens for Raising Healthy Dairy Calves. (Four mimeographed pages issued Jan., 1949, revised Sept., 1949.) U.S.D.A. (Washington, D. C.).

[12] Stoll, N. R.: On Methods of Counting Nematode Ova in Sheep Dung. Parasitol., *23*, (1930): 116-136.

[13] Mayhew, R. L.: Studies on Bovine Gastrointestinal Parasites. IX. The Effects of Nematode Infections During the Larval Period. Cornell Vet., *34*, (1944): 299-307.

[14] Mayhew, R. L.: Studies on Bovine Gastrointestinal Parasites. X. The Effects of Nodular Worm *(Oesophagostomum radiatum)* on Calves During the Prepatent Period. Am. J. Vet. Res., *9*, (1948): 30-34.

[15] Mayhew, R. L.: Studies on Bovine Gastrointestinal Parasites. XII. Additional Infection Experiments with the Hookworm *(Bunostomum phlebotomum)* in the Calf. J. Parasitol., *35*, (1949): 315-321.

The Relation of Silage to Listeriosis in Sheep

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THE DEVELOPMENT of listeriosis and the means of its spread are both unknown.

LITERATURE CITED

Olafson [1] ventured the idea that silage was related to development of the disease but also stated that outbreaks had occurred in animals which had not been fed silage. Graham and his co-workers [2] reported their observations on seven outbreaks of listeriosis in sheep. Four of these flocks had been fed silage. In one outbreak involving 180 feeder lambs fed sorgo silage, 12 died (6.7 %). Their ration was changed so that they received less silage but this had no effect on death losses. Among eight outbreaks of listeriosis in cattle observed by Graham, Levine, and Morrill,[2] silage was fed to cattle in three of the outbreaks. The incidence of disease varied from 2.9 per cent to 13.3 per cent in these three outbreaks. Ryff and Lee [3] reported an outbreak of listeriosis in 240 feeder lambs which had been fed corn silage for three weeks when the first cases began to develop. In this outbreak, 9 lambs died in one week. Jensen and Mackey [4] made observations on 39 outbreaks during two feeding seasons. Sheep were involved in seven of the outbreaks, with an average morbidity of 8.9 per cent (ranging from 3.3 to 15 %). Cattle were involved in 32 outbreaks with an average morbidity of 1.3 per cent (range 0.03 to 7.5 %). Additional information [5] indicated that most of these cases occurred under feedlot conditions in an area where silage is a common feedstuff. In one instance, Jensen [5] reports that a feeder who was giving corn silage to a group of 1,200 lambs discontinued silage and losses subsided within a short period. He again included the silage in the ration and losses again occurred. Silage for the lambs was then discontinued and the losses subsided. Jensen [5] states that he has encountered other outbreaks of listeriosis in which only some were benefited by discontinuation of silage.

The above observations indicate a need for experimental investigation of the relation of silage to listeriosis. The following report represents two attempts in that direction.

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MATERIALS AND METHODS

The plan of experiment 1 was to feed suspected silage to sheep, some of which were also inoculated with *Listeria monocytogenes*. Other sheep, previously fed silage during an outbreak of listeriosis, were inoculated with *L. monocytogenes* and fed a ration of hay and grain.

The corn silage used in this experiment came from a pit silo and was two years old. Silage from this pit silo had been fed to feeder lambs and a mild outbreak of listeriosis had occurred. Details of the outbreak are briefly as follows. The duration of the outbreak was about nine days and the total loss from listeriosis was about 1 per cent. On the premises of the feedlots were several pens holding from one thousand to two thousand sheep. Listeriosis occurred mainly in four of the pens. The sheep in these pens had been fed the silage for about two weeks prior to occurrence of the first case. The disease developed first in those sheep which had been fed more silage for a longer time. For example, pen A contained 2,000 lambs fed corn silage for two weeks. During the outbreak, 38 (2 %) died. In pen B where 1,400 lambs were fed the smaller quantity of silage for two weeks, 20 (1.5 %) died during the nine days of the outbreak. In a third pen of 2,900 lambs, the same ration of corn silage was fed as for pen A but it was begun a few days later. In this pen, 26 (1 %) lambs died.

The 28 sheep used in these experiments were purchased from the feeding establishment which also supplied the silage.

Twelve sheep which were held at the feedlots only a few days before shipment to the laboratory comprised one experimental group. These sheep were fed silage and exposed to *L. monocytogenes* by one of three methods: intracarotid, intravenous, and intrarumen. The dose for intracarotid exposure of sheep was 0.05 ml. of a 24-hour tryptose broth culture per pound of body weight. The dose for intravenous and intrarumen exposure was 0.1 ml. per pound of body weight. Silage was fed twenty days prior to and thirty days after exposure.

A second group of sheep came from pen B soon after the episode of listeriosis in that pen. Eight of these were exposed to *L. monocytogenes*. Three were held as uninoculated controls until well after experimental observations were concluded, at which time they were exposed to *L. monocytogenes* by intracarotid inoculation and their susceptibility to infection established (2 died). A third group of 5 sheep received silage but were not exposed to the disease.

The corn silage used in experiment 2 came from a lot that had been fed to feeder lambs. It was good in quality and made from relatively immature corn ensilated in a pit. A mild outbreak of listeriosis had occurred among 3,200 lambs fed this silage. The mortality during the eight weeks of feeding continued at a rate of 0.17 per cent per week. This mortality was somewhat greater than prior to silage feeding. Still greater mortality was noticed after the animals had been on silage for about five weeks. An experiment was outlined to continue silage feeding to 1,800 lambs for two weeks. Silage was completely eliminated from the ration of the remaining 1,100. During the next two weeks, the mortality among the lambs getting silage increased to 0.9 per cent per week, while death loss in the group of 1,100 decreased to 0.14 per cent weekly. A marked decrease in mortality resulted following the elimination of silage from the ration of 1,800 lambs. During the seven weeks immediately following silage feeding, 0.1 per cent of the animals continued to die with disease in this group as compared with 0.05 per cent in the group of 1,100. Nine yearling ewes were obtained from a farm that had never experienced listeriosis. These were used for experiment 2. The animals were exposed to *L. monocytogenes* by three different routes: intravenous, intrarumen, and intranasal. Three animals were used for each type of exposure. The following dosages were used: 0.1 cc. of a 24-hour tryptose broth culture of *L. monocytogenes* per pound of body weight for intravenous and intrarumen exposure, and 4 cc. for intranasal exposure (2 cc. into each side of nose). Corn silage was fed for three weeks before and three weeks after exposure. Alfalfa hay was fed subsequently.

All sheep were fed, in addition to hay and corn silage, a daily ration of 0.22 lb. corn, 0.22 lb. barley, 0.22 lb. beet pulp, 0.25 lb. soybean meal, and 0.03 lb. bone meal.

Two cultures of *L. monocytogenes* were used in these trials. Culture 136 had been isolated four years before from the brain of a sheep. For three years, it was capable of killing sheep upon carotid exposure[6] but had failed to kill sheep for one year prior to these experiments. Culture 1501-2 was isolated from the brain of a sheep during the outbreak on the premises from which the silage and sheep for experiment 1 were obtained. Intracarotid injection of culture 1501-2 has generally caused fatal listeriosis in about 50 per cent of sheep exposed. Another difference between the two cultures was their virulence for rabbits. Intravenous inoculation of culture 136 had killed rabbits in three to four days (2.5 ml. of 24-hour broth culture) and culture 1501-2 killed them in two days.

Prior to exposure and for ten weeks after exposure, the sheep were carefully watched for symptoms of disease. Counts of leukocytes and rectal temperatures were made at intervals of two to four days by methods previously described.[6] Blood was cultured for evidence of bacteremia by placing

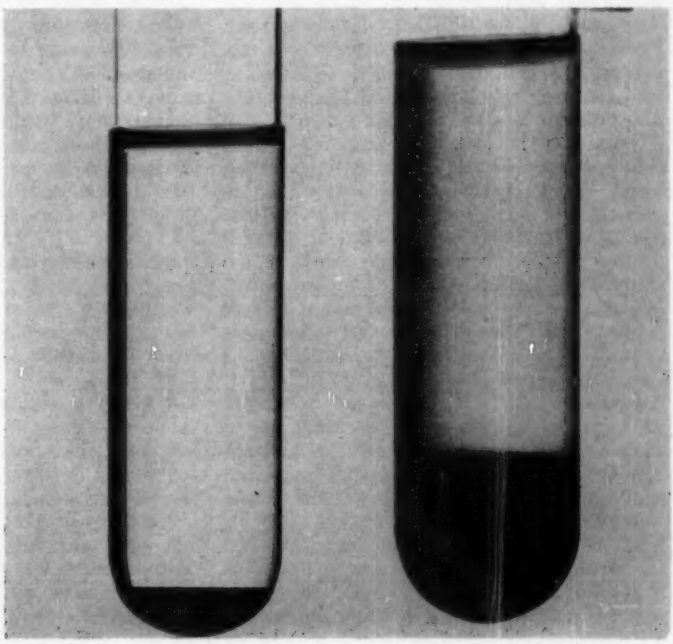


Fig. 1—Hemolysis, with growth of Listeria. Negative culture at left. Positive culture at right. Note zone of hemolysis above layer of blood cells.

1 ml. of fresh blood in each of four tubes of tryptose broth medium to which sodium citrate was added as an anticoagulant. After twenty-four to forty-eight hours' incubation, a narrow zone of hemolysis at the interface between blood and medium developed with growth of *L. monocytogenes* (fig. 1).

RESULTS

No typical organisms were seen in Gram stains of direct smears of the silage. Emulsions of the ground silage were diluted and plated on tryptose-glucose agar and potassium tellurite agar.[5] No typical colonies were seen.

Fatal listeriosis was induced in 4 of the 29 sheep exposed to *L. monocytogenes* (tables 1 and 2). All of those which died received culture 1501-2. Two of them were inoculated via the carotid artery. One had been fed the suspected corn silage continuously and the other had not. Two others that died (sheep 311 and 655) had received an intravenous inoculation of the culture. Each of them was fed different silage. This result was unusual, since we have heretofore been unable to induce fatal listeriosis by intravenous inoculation.

In general, all of the sheep showed an increase of neutrophils and a decrease of lymphocytes following exposure. The degree of this reaction appeared to be correlated with the culture of organism that was used. Culture 1501-2 was associated with a slightly less marked leukocyte reaction than that following exposure to culture

TABLE I—Comparison of Symptoms Following Experimental Exposure to Listeria Monocytogenes (All Received Corn Silage (lot 1) for at Least Two Weeks Before Exposure)

Sheep No.	Culture No.	Method of exposure	Neutrophil increase (days)	Lymphocyte decrease (days)	Duration of symptoms pyrexia 1 degree or more (days)	Bacteremia 10 min.	1 day	2 days	3 days	5 days	Remarks
Corn silage fed for 30 days after exposure											
302	136	Intra-carotid	6	1	9		+		+		
313			9	1	9		+		+		
315	1501-2		6	0	0		+		—		Symptoms in 41 days
316			6	0	6		—		—		Died in 22 days
304	136	Intra-venous	1	1	9		+		—		
314			6–9	1	6		—		—		
310	1501-2		1	0	9		—		+		
311			3	9	6		—		—		Died in 36 days
305	136	Intra-rumen	21+	1	6		+		—		
317			1	1	9		—		—		
306	1501-2		7	0	3		—		—		
307			0	0	0		—		—		
No corn silage fed after exposure											
683	136	Intra-carotid	6	1	4	+	+	+	+	—	
686			6	0	8	+	+	—	+	+	
685	1501-2		8	1	6	+	+		—	—	
676			1	1	0	+	+	—	—	—	Died in 29 days
681	136	Intra-venous	0	1	2	+	+	—	—	—	
684			4	1	6	+	—	—	—	+	
677	1501-2		1	0	1	+	—	—	—	—	
680			1	3	1	+	—	—	—	—	

TABLE 2—Comparison of Symptoms Following Experimental Exposure to Listeria Monocytogenes (strain 1501-2) (All Received Corn Silage (lot 2) for Three Weeks Before and Three Weeks After Exposure)

Sheep No.	Method of exposure	Neutrophil increase (days)	Lymphocyte decrease (days)	Duration of symptoms pyrexia 1 degree or more (days)	Bacteremia 1 hr.	6 hr.	1 day	2 days	3 days	5 days	6 days	7 days	Remarks
655	Intravenous			6	+		−	−	−	−		−	Died in 21 days
663				10	+		+	−	−	−		−	
667				12	+		+	+	+	+	+	+	Sick at 38 days and recovered
658	Intrarumen	2	0	2		−	−	−	−	−		−	
669		2	1	2		−	−	+	−	−		−	
670		2	0	2		−	−	−	−	−		−	
652	Intranasal	1	1	3		−	−	+	+	−	−	−	
654		2	1	3		−	−	−	−	−		−	
665		2	1	5		−	−	−	−	−		−	

136. Culture 136 caused a somewhat more prolonged elevation of temperature.

Observations on sheep 316 fed silage and inoculated with culture 1501-2 into its carotid artery were typical of the results in a fatal case (graph 1). There was a preliminary increase of neutrophils and an elevation of temperature. Both subsided to normal levels in about six days. The elevation of neutrophils prior to death, twenty-two days after exposure, indicated active infection. The reaction of sheep 302 may be regarded as characteristic for those animals which did not die from the experimental exposure. This animal received culture 136 in its carotid artery and was also fed corn silage. There was an increase of neutrophils, decrease in lymphocytes, and temporary increase of temperature with return to normal (graph 2).

A peculiar reaction was noted in sheep 315 which was fed silage and which received culture 1501-2 via the carotid artery. There was a temporary increase of neutrophils, but lymphocytes and body temperature remained essentially unchanged following exposure. Forty-one days after inoculation, there was clinical evidence of paralysis of one ear. At this time there was also an elevation of neutrophils. Both symptoms subsided and the animal was subsequently normal (graph 3).

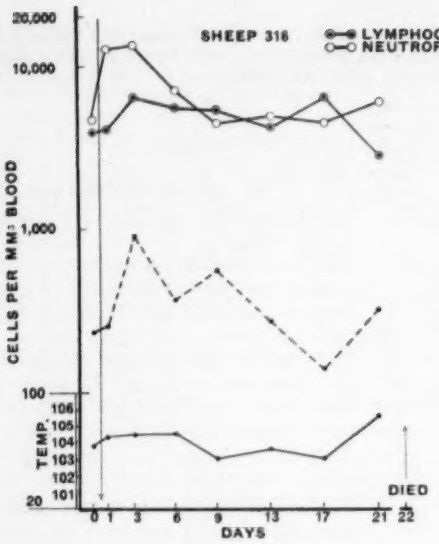
Clinical evidence of infection was observed in sheep 667 fed silage and inoculated intravenously. This animal had a bacteremia for seven days following the exposure. Thirty-eight days after inoculation the sheep appeared to be unsteady and had a slight incoördination of movement. It tended to circle to the right. There was a definite strabismus of the left eye. The optical axis of this eye was pointed laterally when compared with the right eye. There was no evidence of an increase of neutrophils. Temperature was 105.2. During the subsequent three days the symptoms gradually disappeared and the animal recovered completely.

Two sheep (305 and 317, table 1), which were fed silage continuously, received culture 136 in their rumens and developed a diarrhea of short duration on the seventh postinoculation day. Inappetence was also noted for two or three days. These symptoms were not correlated with any characteristic changes of leukocytes or body temperature.

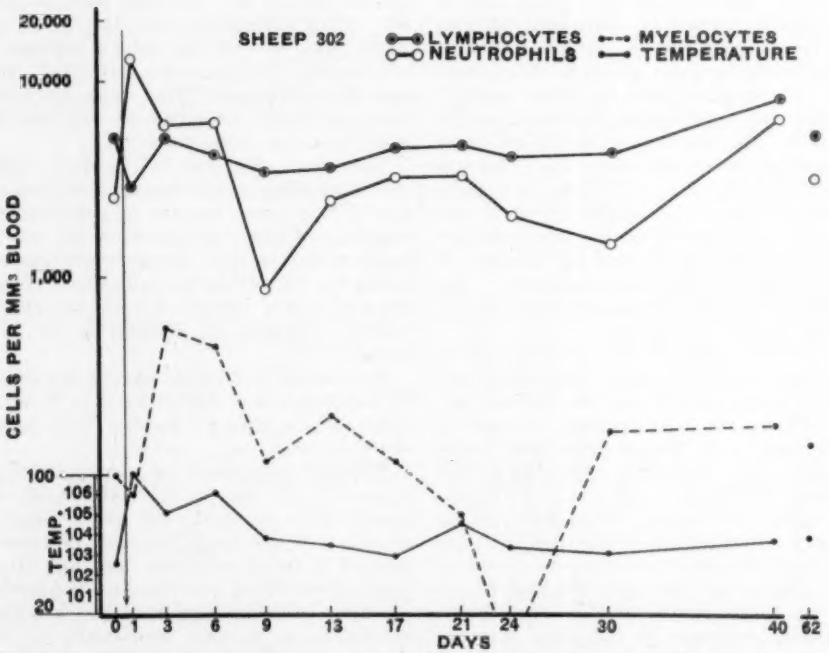
Five sheep fed silage during the experimental trials, but not exposed to *L. monocytogenes*, remained normal and apparently healthy.

Typical symptoms of acute listeriosis appeared in sheep 311, 316, and 655 twenty-four to thirty-six hours prior to death with the usual marked depression, unilateral facial paralysis, and loss of corneal reflex. Sheep 676 developed symptoms six days prior to death and had a definite torticollis, as is seen occasionally in field cases where the course is prolonged.

The bacteremia of sheep exposed in a like manner was essentially similar regard-



Graph 1—Observations on sheep 316 after intracarotid exposure to Listeria monocytogenes (strain 1501-2). Note apparently normal leukocyte values and temperature from ninth to seventeenth day after exposure.



Graph 2—Observations on sheep 302 after intracarotid exposure to Listeria monocytogenes (strain 136). Note temporary reaction for six days after exposure.

less of silage feeding. The presence of the organism in the blood was generally more persistent in sheep exposed via the carotid artery with culture 136 (table 1). Only 2 of 7 sheep which received *L. monocytogenes* in the rumen had the organisms in the blood twenty-four and forty-eight hours later. A bacteremia developed in 1 of 3 sheep exposed intranasally. The organism was isolated at forty-eight and seventy-two hours following exposure.

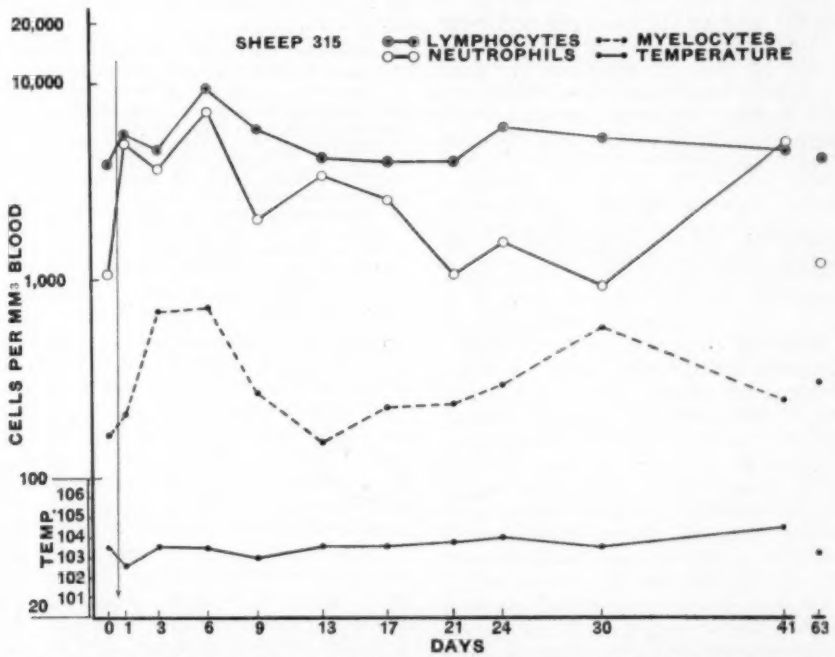
Eight sheep not fed silage after exposure, whose blood was cultured frequently between the sixth and thirty-third day after exposure, remained negative.

In general, the continuous feeding of corn silage appeared to have no effect in prolonging the bacteremia.

The necropsy, bacteriological and histological findings on sheep 311, 316, 655, and 676 were essentially similar and showed no noteworthy differences from ordinary experimental listeriosis of sheep.

Discussion

The results of these experiments were somewhat disappointing in that they did not give a clear-cut answer to the relation of silage to experimental listeriosis. However, they did provide some information. Fatal listeriosis following intravenous exposure of 2 lambs fed the suspected corn silage was new in our experience.[6] Most other workers have not induced fatal listeriosis with a single intravenous dose of the culture in the sheep. Graham *et al.*[2] report death of 1 lamb eighteen days after intravenous inoculation with a heavy suspension of *L. monocytogenes*. These workers also caused death of 7 young lambs (20 to 28 lb. in weight) and 5 of 6 larger sheep (73 to 139 lb. in weight). The amount of Listeria injected was relatively large. The finding of bacteremia in 2 of 7 sheep that received the Listeria in their rumen, while being fed silage continuously, indicates a possible portal of entry. Benjamin[7] fed



Graph 3—Observations on sheep 315 after intracarotid exposure to Listeria monocytogenes (strain 1501-2). Note temporary reaction of leukocytes for seventeen days after inoculation and again at forty-one days. Subsequent complete recovery.

silage from infected premises to sheep, in conjunction with Listeria organism in their drinking water. Since no clinical symptoms of listeriosis developed in either set of experiments, doubt may be cast on the importance of oral infection.

It would appear that silage coming from a farm where listeriosis had occurred did not render sheep susceptible to listeriosis under the conditions of our experiment. It would also appear that sheep fed silage during an outbreak of listeriosis were not made more susceptible to experimental listeriosis when removed from that environment and subjected to exposure.

Our inability to demonstrate *L. monocytogenes* in corn silage fed to sheep undergoing an outbreak of listeriosis confirms similar trials of Benjamin[7] in Colorado. She[7] also added Listeria to filtered juice extracted from the suspected silage and found survival for only twenty-four hours in a sample kept at —13 C.

The temporary symptoms of strabismus, elevation of temperature, and ataxia in sheep 667, and ataxia, paralysis of the ear, and leukocytosis in sheep 315 indicate that not all cases of listeriosis are fatal. Such clinical cases could be overlooked in field outbreaks. The existence of such mild or asymptomatic cases in field outbreaks has been suspected.[9]

SUMMARY

Sheep were fed corn silage from two specific lots which were associated with an outbreak of listeriosis. They were then exposed to *Listeria monocytogenes*. Two cultures, one of high and one of low pathogenic ability, were used. Exposures were made via the carotid artery, intravenously, intranasally, and into the rumen. Eight of the 29 sheep received no silage after exposure. Observations were made for symptoms following exposure.

There was no clear-cut effect of continuous feeding of silage on development of listeriosis in the sheep. The feeding of silage as a preparatory treatment before exposure did not reduce the resistance to fatal listeriosis except in 2 cases, the significance of which is questionable.

References

[1] Olafson, P.: Listerella Encephalitis (Circling Disease) of Sheep, Cattle and Goats. Cornell Vet., *30*, (1940): 141-150.

[2] Graham, R., Levine, N. D., and Morrill, C. C.: Listerellosis in Domestic Animals. Univ. of Illinois Agric. Exper. Sta. Bull., No. 499, (1943): 99.

[3] Ryff, J. F., and Lee, A. M.: Listerellosis. Report of an Outbreak in Wyoming Sheep and Preliminary Experimental Therapy in Rabbits. Am. J. Vet. Res., *9*, (1948): 147-151.

[4] Jensen, R., and Mackey, D. R.: Listerellosis in Cattle and Sheep. J.A.V.M.A., *114*, (1949): 420-424.

[5] Jensen, R.: Personal communication.

[6] Olson, C., Cook, R. H., and Blore, I. C.: The Reaction of Blood Cells in Experimental Listeriosis in Sheep. Am. J. Vet. Res., *11*, (1950): 29-40.

[7] Benjamin, M. M.: Personal communication.

[8] Gray, M. L., Stafseth, H. J., and Thorp, Frank, Jr.: The Use of Potassium Tellurite, Sodium Azide, and Acetic Acid in a Selective Medium for the Isolation of *Listeria Monocytogenes*. J. Bact., *59*, (1950): 443-444.

[9] Olson, C., Cook, R. H., and Bagdonas, V.: An Attempt to Immunize Sheep During an Outbreak of Listerosis. Am. J. Vet. Res., *12*, (1951): 306-313.

Prolonged Insulin Hypoglycemia in Sheep

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Literature Cited

Until the recent work of Reid,[8, 9] only a few reports of insulin hypoglycemia in sheep could be found in the literature.[1, 2, 4, 10] These were inconclusive as to the effect of insulin upon the glycemic level and the subsequent response of the animal to low levels of blood glucose. Jasper[5, 6] has indicated that in the case of cows, symptomatology is dependent not upon degree of hypoglycemia alone but, to a large extent, upon duration of hypoglycemia as well.

The present report concerns an investigation into the symptomatology of prolonged insulin hypoglycemia in ewes.

Materials and Methods

Eight mature crossbred whiteface ewes were used for the experiment. Two were nonpregnant, 5 were pregnant, being from twenty-one to eighty-five days from term, and the status of 1, presumably pregnant, was not finally determined. Hypoglycemia was induced initially by 1 unit per kilogram of regular insulin injected intravenously (i.v.), and 1 unit per kilogram of protamine insulin injected subcutaneously following a 24-hour fast. An additional i.v. injection of regular insulin was made five to seven hours later and protamine insulin was again injected about the tenth hour. Further injections were occasionally made when warranted by the response of the individual animals. True blood sugar values were determined by the method of Nelson.[7]

Results

Considerable individual variation in the reaction of the animals to injected insulin was observed. In animals E, F, and G, difficulty was encountered in maintaining a persistent low level of blood glucose. In the others, a minimum range of between 5 and 12 mg. per 100 cc. of blood was maintained for an appreciable period of time.

A summary of the results is presented in table 1. Figures 1 and 2 illustrate two glucose curves, the severity of symptoms being shown also on an arbitrary scale.

An attempt was made to classify the symptoms into the four stages proposed by Reid.[9] True convulsive activity was not noted, though marked tremors, shaking, and other abnormal muscular responses were observed during earlier stages. The most severe reactions were characterized more by near coma than by convulsions. Consequently, no stage 4 responses were recorded.

By references to table 1, a fairly uniform pattern of progression can be seen. Stage 1 symptoms, manifested principally by lassitude, quietness, and sometimes by marked lethargy, became apparent, at least mildly, soon after establishment of hypoglycemia. Blood glucose values at this stage varied between 5 and 20 mg. per 100 cc. Animals remained in this stage between two and twenty-one hours, the average being fourteen hours.

Stage 2 symptoms were manifest by extreme lethargy, disinclination to stand or move about, tremors or severe muscular twitching, opisthotonus and other abnormal postures or gaits, and almost always impaired vision progressing to blindness. Appetite usually failed completely during this stage. Salivation, lacrimation, and continuous masticating movements were often present. Standing sheep tended to hold the head low. All the symptoms described were not necessarily present in any 1 animal. Blood glucose values varied between 6 and 23 mg. per 100 cc. of blood but were usually between 6 and 10 mg. per 100 cc. This stage was shorter than stage 1, in 3 cases being only an hour or two, and in 1 case, fourteen hours. The average duration was six hours.

Stage 3 symptoms were observed in 4 animals. They were differentiated from stage 2 symptoms chiefly by general increased severity and inability to stand. Respirations tended to become very irreguar and shallow. Duration of stage 3 was either fairly short or deliberately modified after a few hours by glucose injections. Glucose levels during this stage varied between 6 and 11 mg. per 100 cc. of blood, in 1 case reaching 19 mg. per 100 cc. Development of stage 3 symptoms was not paralleled by blood glucose levels lower than those in stage 1 or 2 but appeared to reflect an extension of the hypoglycemia.

Prompt remission of symptoms following administration of glucose occurred only in ewe C after two hours of stage 3

From the School of Veterinary Medicine, University of California, Davis.

TABLE 1—Individual Variation in the Reaction to Injected Insulin

Ewe	Hours post-injection	Blood glucose (mg. per 100 cc. of blood)	Symptoms	Stages
A	2 to 23	8 to 15	Very quiet; slight head tremors after 2 hours.	1
47 days Antepartum	25 to 34.5	6.6 to 18.5	Fell, but could soon stand again; tremors of head, neck, ears; blind.	2
	34.5 to 55	14.2 to 73	Prefers to lie down; appetite returning; blind for more than 48 hours, posthypoglycemia.	1
B	2 to 23	6 to 19	Slightly more quiet than normal.	1
78 days Antepartum	24 to 38	6 to 19	Marked tremors; blind, butted against wall, went down.	2 and 3
	40 to 60	27 to 97	Slow gradual recovery; sight returned slowly by 48 hours posthypoglycemia.	1
C	5 to 25	4 to 11.9	More quiet than normal, definitely prefers to rest.	1
76 days Antepartum	25.5 to 27	6.6 to 7.4	Down, unable or refuses to rise; few tremors; blind. (jumped up and walked naturally 5 min. after 75 ml. of 50 % glucose, i.v.)	3
	27 to 38	4.2 to 14.1	Lethargic; refuses hay but improving steadily.	1
	40.5 to 44	12.8 to 45.5	Normal again.	
D	3 to 17	5.5 to 21.5	Temporarily more quiet at initial hypoglycemia.	1 or normal
	19	7.0	Down, but will stand; unsteady gait; head and neck tremors.	2
	20 to 28	6.5 to 11.5	Down, unable or refuses to rise; slight tremors; marked hypersalivation; will not eat; blind; shallow respiration; almost comatose. 22 ml. of 50 % glucose i.v. at 23 hours and 25 ml. i.v. at 28 hours.	3
	28 to 36	70 to 88	Slight tremors; stands around but walks some; hypersalivation; blind.	2
	37 to 41+	87.5	Down; rapid, forced nibbling movements on floor, abrosed lips, excessive hypersalivation and serous nasal discharge. 125 ml. C.G.P.* solution i.v. Found dead with severe pulmonary edema 2.5 hours later.	3
E Open	35 to 48	10 to 20.0	Depression only; very resistant to insulin.	1
F	3 to 5	12 to 15	Stiff awkward gait; twitching eyelids and ears.	1
	6 to 31	11.5 to 37	Quiet.	1
Open	32 to 40	6.5 to 23.0	Very quiet; head tremors; unsteady gait; occasional nibbling movements against concrete floor at lowest glucose level.	2
	41 to 49	12 to 19.5	Impaired vision; unable to stand; severe muscular twitching, shaking of head, or opisthotonus; bites vigorously at the floor; pushes in circles upon knees and hind legs; down again.	3
	51 to 72	23 to 72	Continued opisthotonus until hour 72; full return of vision, appetite, and locomotion.	1
G				
17 days Antepartum	13 to 26	10 to 21	Very quiet; depression; suggestion of tremors.	1
	28 to 38	10 to 12	Will stand with much urging; moves head in circles, up or down, or very near to the floor; nearly blind, respirations fast and irregular.	2 and 3
	39 to 55	15 to 29	Slow improvement from advanced stages; temporary twitching at hour 45.	1
H	10 to 18	7 to 14	Slight depression only.	1
85 days Antepartum	19 to 33	12 to 53	Approximately normal.	
	35	11	Marked depression; head held low; will stand with *much* urging.	2
	37 to 41	7 to 11	Unable or barely able to stand; head held in rigid opisthotonus or very low; blind. 50 mg. of 50 % glucose i.v. at hour 40 gave temporary improvement.	3
	43 to 44	10	Lying flat and very limp; rapid irregular respirations; blind. 50 ml. of 50 % glucose i.v. at hour 44 initiated progressive improvement.	3
	45	27	Improved; attempted to rise but could not.	3
	47 to 55	7.5 to 72	Steady improvement, even at temporary blood glucose level of 7.5; marked hypersalivation.	2
	72 to 97	35 to 58	Slow improvement; stands around by itself. Still blind at 97 hours though appetite had returned. Sight normal at about 120 hours.	1

* Diphosphocalcium gluconate (Haver-Glover Laboratories).

symptoms. In ewe H, temporary improvement followed 50 ml. of 50 per cent glucose, i.v., at forty and forty-four hours, hypoglycemia reoccurring after each injection. Between fifty and 100 hours postinjection, the glucose level varied between 36 and 68 mg. per 100 cc. of blood while symptoms abated slowly, vision being restored finally between one hundred and 120 hours. In ewe D, repeated glucose injections and a persistant glucose level of over 80 mg. per 100 cc. failed to bring about recovery after a relatively short period of stage 3 symptoms. Spontaneous recovery was allowed in the other ewes.

DISCUSSION

Levels of 5 mg. per 100 cc. of blood or less were recorded in only 3 sheep. These levels were not maintained for an extended period of time. Temporary falls to this level did not appear to immediately promote increased severity of symptoms, but the results do suggest that prolonged periods at this level would be serious. This observation corroborates that of Reid[9] who states, "It appears that depression of the venous blood glucose below 5 mg. per cent for a reasonable period in any normal sheep will be associated with hypoglycemic disturbances, the severity of which will depend on the degree of hypoglycemia and the period for which it is maintained."

Though the 5 mg. per 100-cc.-level appeared to be critical in the sense, as suggested by Reid, that it was very difficult to reduce the blood glucose below this level, it did not seem to be quite so critical as suggested regarding the development of symptoms. Indeed, the severe symptoms observed in these experiments almost without exception occurred at higher glucose levels of between 6 and 12 mg. per 100 cc. of blood. These findings are similar to the case in cows[5,6] in which it was observed that prolonged hypoglycemia, even if not extreme, will produce serious symptoms. The 5 mg. per 100-cc.-level may then in reality be a level below which it is very difficult to reduce the blood glucose and at or below which the time required for development of symptoms is greatly reduced.

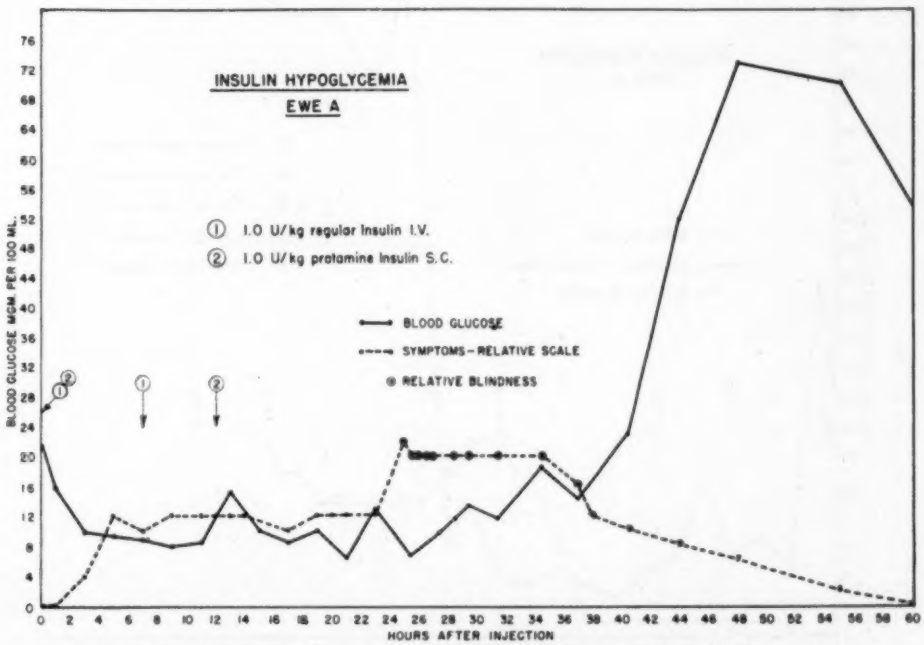

Fig. 1—Insulin hypoglycemia in ewe A. Relative blindness is indicated by completeness of outer circle.

Recovery from stages 2 and 3 reverted in general through the earlier stages but was not at all prompt. Hyperglycemic levels were usually attained following cessation of insulin influence. Even so, complete recovery from symptoms was delayed many hours and in the case of ewe D, death eventually ensued from pulmonary edema though only a relatively short period of hypoglycemia was endured.

Assuming that symptoms are due to inadequate glucose oxidation by the neurons,[8] delayed clinical recovery after prolonged hypoglycemia may be the result of an essential period of recovery required by the damaged cells. Clinical observations indicated that vision was one of the last functions to return to normal.

Since anorexia appears soon after initiation of hypoglycemia and may persist for some time after normal or high blood glucose levels are restored, loss of appetite in clinical ketosis may be the direct result of an already existent hypoglycemia.

SUMMARY AND CONCLUSIONS

1) Eight ewes were subjected to prolonged insulin hypoglycemia and the symptoms were recorded.

2) Symptoms were classified according to severity into three stages.

3) Severity of symptoms was dependent not only upon depth of hypoglycemia but to a large degree upon duration as well. Little or no difference in glycemic levels was apparent between the different stages of symptoms observed, duration of hypoglycemia being the determining factor.

4) Marked symptoms often persist for several hours or days following spontaneous or induced return to normal or hyperglycemic levels.

5) In the ewe, it is difficult to produce a hypoglycemia below 5 mg. per 100 cc. of blood.

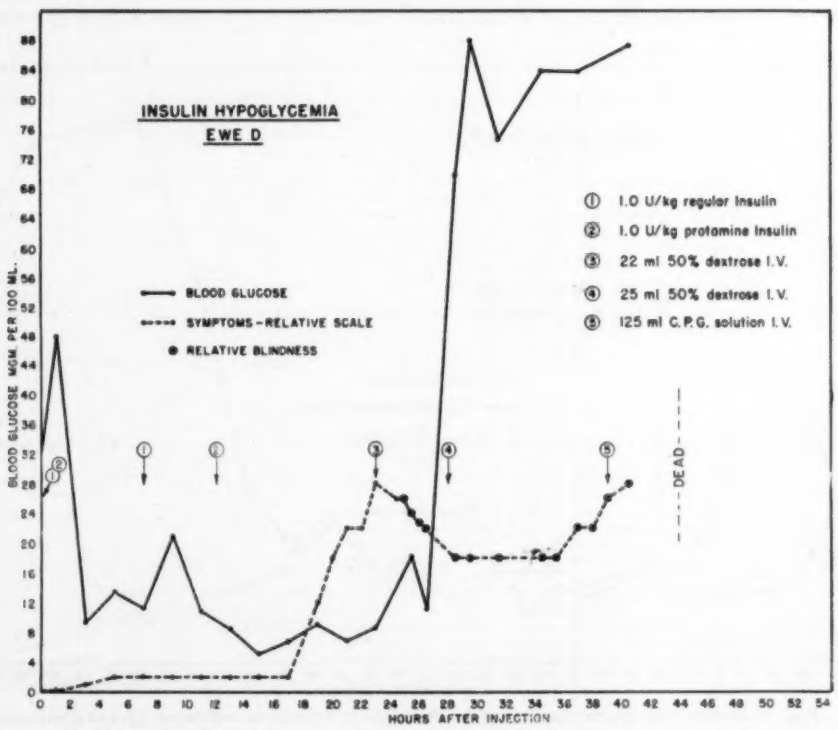

Fig. 2—Insulin hypoglycemia in ewe D. Blindness is indicated by outer circle.

References

[1] Bodansky, A.: Antagonistic Effects of Insulin and Thyroxin. Proc. Soc. Exptl. Biol. Med., *20*, (1923): 538-540.

[2] Bodansky, A.: Effects of Dosage and Previous Diet on Blood Sugar Curves in Sheep After Intravenous Administration of Insulin. Proc. Soc. Exptl. Biol. Med., *21*, (1924): 416-417.

[3] Himwich, H. E., Bowmen, K. M., Wortis, J., and Fazekos, J. F.: Metabolism of the Brain During Insulin and Metrazol Treatments of Schizophrenia. J. Am. M. A., *112*, (1939): 1572.

[4] Hitchcock, M. W. S., and Phillipson, A. J.: The Tolerance of Sheep to Low Concentrations of Blood Sugar. J. Physiol., *105*, (1946): 42.

[5] Jasper, Donald E.: Heptac Changes in the Bovine During Pregnancy and Ketosis. Doctor of Philosophy Thesis, University of Minnesota, St. Paul. 1947.

[6] Jasper, Donald E.: Acute and Prolonged Insulin Hypoglycemia in Cows. Am. J. Vet. Res., *14*, (1953):

[7] Nelson, Norton: A Photometric Adaption of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., *153*, (1944): 375-380.

[8] Reid, R. L.: Studies on Carbohydrate Metabolism of Sheep. III. The Blood Glucose During Insulin Hypoglycemia. Austral. J. Agric. Res., *2*, (1951): 132-145.

[9] Reid, R. L.: Studies on Carbohydrate Metabolism of Sheep. IV. Hypoglycemic Signs and Their Relation to Blood Glucose. Austral. J. Agric. Res., *2*, (1951): 146-157.

[10] Strand, R., Anderson, W., and Allcroft, W. H.: Further Studies on the Lactic Acid, Sugar, and Phosphorus of the Blood of Ruminants, (a) Following Adrenalectomy and (b) After Intravenous Injections of Insulin. Biochem J., *28*, (1934): 642-649.

Electrocardiographic Changes in Vitamin E-Deficient Lambs

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Literature Cited

Culik *et al.*[1] and Bacigalupo *et al.*[2] have reported that vitamin E deficiency regularly produces degeneration of skeletal and heart muscle of young lambs. Similar cardiac effects have been observed in cattle by Gullickson and Calverley[3] and in rabbits by Gatz and Houchin[4] and Bragdon and Levine.[5] Gullickson and Calverley[3] found that the main electrocardiographic * change in vitamin E-deficient cattle was an increase in the PR interval with terminal effects indicating disorganization of the heart beat in a few instances. Gatz and Houchin[4] observed that records of deficient rabbits, as compared with those of normal animals, showed right axis deviation, notching of the T wave frequently occurring in leads 1 and 2, and inversion of T in lead 3. Bragdon and Levine,[5] however, reported later that electrocardiograms of vitamin E-deficient rabbits had elevated ST segments and inverted T waves in lead 2, which were observed only during the latter stage of the deficiency.

In monkeys, Filer *et al.*[6] found that chronic vitamin E deficiency reduced the amplitude of the R and T waves and inverted the T waves. He found these changes quite similar to those occurring in vitamin B_1 deficiency.

The purpose of the present investigation was to determine by means of electrocardiograms whether any functional change of the heart was associated with the cardiac lesions produced in the lamb by vitamin E deficiency.

Experimental Data

Electrocardiographic data reported in this paper were obtained from 20 male Shropshire lambs used in the experiment carried out by Bacigalupo *et al.*[2] and Culik *et al.*[1] The animals were fed *ad libitum* a purified vitamin E-free liquid diet similar to that described by Luecke *et al.*[7] but in which *alpha*-tocopherol acetate was not added, and the only source of carbohydrate and fat were, respectively, glucose and a molecular distilled lard of a very low tocopherol content.* Three lambs served as controls. These animals received the same diet as the experimental lambs except that 100 mg. of *alpha*-tocopherol acetate were given orally every other day. Recovery from vitamin E deficiency was attempted in 3 of the lambs deficient in vitamin E.

All electrocardiograms (ECG's) were recorded in the evening before the last feeding. The first ECG's were taken within five days of the beginning of the experiment when the lambs were 9 to 10 days old. Subsequent ECG's were taken every seven days until the end of the experiment, which varied from twenty-five to eighty-one days. The three standard dipolar (Einthovan) leads were

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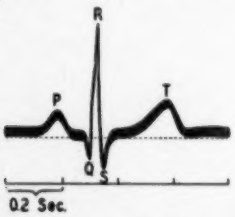
* An electrocardiogram (ECG) is a recording of the electrical effects accompanying the heart beat as these are lead off from various points on the surface of the body. These are recorded as a succession of waves as the several regions of the heart become active. The apparatus employed for making these recordings is called an electrocardiograph. The waves have been arbitrarily designated as P, Q, R, S and T, the order in which they occur during each heart beat. For purposes of illustration, the form of the principal waves from a human heart beat is shown below.



Waves above the dotted isoelectric line are designated as positive deflections; those appearing below the line are negative. The P wave is formed during atrial activity; Q, R, S, and T arise during ventricular activity.

Electrocardiograms are recorded in such a manner that it is possible to determine the length of time necessary for the completion of various phases of the cardiac cycle. The interval from the beginning of the P wave to the beginning of the QRS complex (PR interval) represents the time occupied by atrial activity, while that from the beginning of the QRS to the end of the T wave (QT interval) is the time occupied by ventricular activity. Similarly, the ST segment represents that portion of the record from the end of QRS to the beginning of the T wave. The RR interval is the time from the beginning of one QRS group to the next succeeding one.

It is possible, also, to determine from the electrocardiogram the relative height or potential of the various waves. Variations in the length of the intervals and, under certain circumstances, the potential of the waves may indicate abnormalities in cardiac function. To the uninitiated, electrocardiograms from animals may appear different from those of the human subject, but the resemblance is sufficiently close to permit use of the same nomenclature for the various waves and intervals.

* Distillation Products Inc., Rochester 3, N. Y.

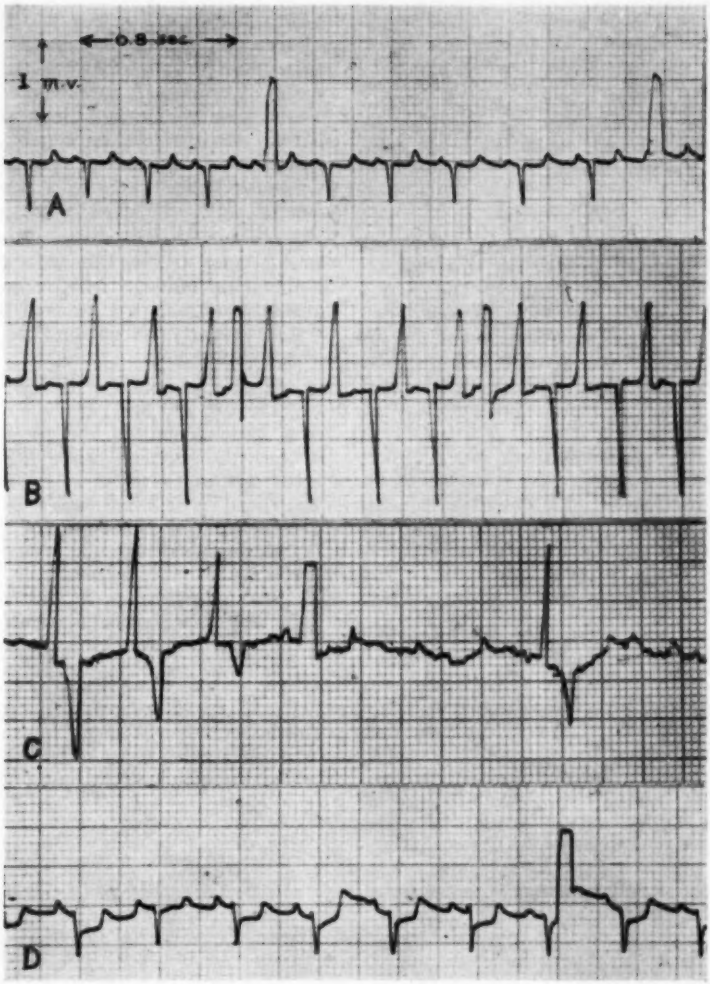


Fig. 1—Records from 4 different lambs illustrating certain of the ECG changes accompanying vitamin E deficiency.

A) Typical normal tracing from a control animal (lead 2 from lamb 40).

B) Tracing showing QRS and T wave changes in a lamb (lead 1 from lamb 48) after twenty-eight days on the E-deficient diet and recorded twenty-four hours before death.

C) Tracing showing evidence of periodical disorganization of the cardiac impulse interspersed with waves of variable voltage from an animal (lead 2 from lamb 39) after twenty-three days on the E-deficient diet and recorded eighteen hours before death.

D) Tracing (lead 2 from lamb 19) showing depression of the ST segment. This record was taken after sixty-seven days on the vitamin E-deficient diet, twenty-four hours before the death of the animal.

successively recorded by means of a direct recording electrocardiagraph.** The areas of application of the electrodes were situated a little above the knees in the front legs, and a little above the hocks in the rear legs. In order to establish intimate contact of the electrodes with the skin, the wool was clipped and the skin rubbed with ethyl ether followed by electrode paste.

RESULTS

The ECG of Control Lambs.—The serial ECG's of control animals in this experiment were essentially uniform (fig. 1 A). The average heart rate was 157 beats per minute (table 1). The durations of the intervals between waves were as follows: PR, 0.08 ± 0.001;† QRS, 0.06 ± 0.001; and QT, 0.23 ± 0.002 second. The potential of the T wave usually ranged from 0.05 to 0.50 mv. In a few instances, the T wave was so small in one of the leads as to be barely discernible. The potential of the largest deflection of QRS ranged from 0.25 to 1.40 mv. P waves were often not perceptible. When present, the potential ranged from an estimated 0.05 to 0.15 mv.

** Cardiotron Model PC-1a. Electro-Physical Laboratories Inc., N. Y.

† Standard error.

The ECG of Vitamin E-Deficient Lambs.—All the lambs considered in this group showed the following clinical signs typical of vitamin E deficiency:[1,2] progressive decrease of the total blood plasma tocopherol, an increase in the excretion of creatine in the urine, and macro- and micropathological changes in skeletal muscles.

Electrocardiograms from these animals showed longer PR intervals than those from control lambs. Usually the PR interval increased from a normal of 0.08 second to 0.10 second and in some cases to 0.12 second (table 1).

Five of the vitamin E-deficient lambs (14, 19, 24, 39, and 48) died suddenly. These animals showed abrupt and striking electrocardiographic changes which occurred within twenty-four hours of death. This would seem to indicate that death may have been due to heart failure. In 3 lambs (14, 24, and 48), these changes consisted of very large voltage increases of the T and QRS waves, particularly the former (fig. 1 B). The T waves consequently assumed a very typical sharp form resembling that of the R waves. The usual potential of

TABLE I—Average Heart Rates, PR, and QT Intervals of Vitamin E-Deficient and Normal Control Lambs

Group	Lamb (No.)	Days on experiment	No. of records	Heart rate	Intervals		Bazett's constant K*
					PR	QT	
Controls	37	81	11	166	0.08	0.22	0.37
	40	78	11	151	0.08	0.23	0.36
	41	78	11	155	0.08	0.24	0.37
	Ave.			157	0.08	0.23	0.37
Vitamin E-deficient lambs	14**	69	1	232	0.06	0.18	0.35
	19**	67	1	150	0.10	0.22	0.35
	24**	55	3	175	0.10	0.22	0.38
	39**	43	6	149	0.10	0.22	0.35
	48**	28	4	200	0.08	0.19	0.34
	34	64	8	155	0.10	0.24	0.37
	38	57	8	176	0.10	0.22	0.36
	42	25	4	138	0.12	0.26	0.39
	43	53	8	139	0.12	0.25	0.38
	44	74	11	137	0.10	0.26	0.39
	45	56	8	131	0.12	0.25	0.37
	46	49	7	115	0.09	0.27	0.37
	47	70	10	132	0.11	0.26	0.38
	49	53	8	103	0.10	0.29	0.38
	12	69	1	150	0.10	0.24	0.38
	32	27	2	158	.....	0.20	0.33
	50	32	5	140	0.08	0.24	0.36
	Ave.			151	0.11	0.23	0.37
Vitamin E-deficient lambs treated with α-tocopherol acetate	42	14	3	139	0.12	0.25	0.39
	49	7	2	73	.....	0.30	0.38
	50	28	5	143	0.10	0.24	0.37
	Ave.			118	0.11	0.26	0.38

* Pardee;[3] ** These animals died suddenly after showing abrupt electrocardiographic changes.

these waves was 0.60 mv., but occasionally it went up to 0.85 and 1.05 mv. Tachycardia, changes in the direction of the various waves, and evidence of disorganization of the cardiac impulse also occurred in lambs 14 and 39 (fig. 1 C). The ECG showed extremely large QRS deflections which were followed immediately by large inverted T waves in all three leads. In lamb 19, electrocardiographic changes occurred which differed from any previously seen. These were marked depression of the ST segment (—0.15 mv.) in leads 2 (fig. 1 D) and 3, variations in the potential and form of the P waves from one cycle to the next, and diphasic T waves in lead 3.

In contrast to the terminal electrocardiographic changes mentioned above, the ECG of 3 other lambs (12, 32, and 50), which had become progressively ill, were similar to the records of the control animals even though the lambs were moribund.

The ECG of Vitamin E-Deficient Lambs Treated With Alpha-Tocopherol Acetate.— It was reported by Bacigalupo *et al.*[2] that *alpha*-tocopherol was an effective treatment in lambs which were not severely affected by vitamin E deficiency. However, this improvement was not reflected in any electrocardiographic change in lambs 42, 49, or 50, which had been supplemented with *alpha*-tocopherol acetate for fourteen, seven, and twenty-eight days, respectively.

DISCUSSION

Lack of information concerning the normal ECG of the sheep, and particularly the lamb, was a serious limitation on this investigation. For this reason, it was considered necessary to obtain further information on the normal ECG of the lamb. This was accomplished by carrying out an electrocardiographic study on a group of 22 healthy lambs raised under normal farm conditions. These were male and female animals of the Shropshire breed. Three serial ECG's were recorded as described previously—the first one, when the average age of the lambs was 17 days (range 5 to 36 days), and the two others two and four weeks later.

It was found in every lamb that no significant changes in the electrocardiographic deflections occurred between 5 and 64 days of age, though there was a small variation in the voltage, duration, and pattern of the ECG's among different individuals. The average heart rate and duration of the various intervals during the observation period were as follows: heart rate, 180 ± 4.33;* PR interval, 0.08 ± 0.001 sec.; QT interval, 0.21 ± 0.006 sec.; and Bazett's constant,[5] 0.36 ± 0.003.** The amplitude of the various deflections was small. The voltage of the largest deflection of the QRS complex was 0.30 ± 0.014 mv. The potential of the P and T waves was 0.071 ± 0.006, and 0.30 ± 0.14 mv., respectively. Changes in the heart rate affected the duration of the QT interval but did not change the duration of the PR interval.

These figures agree with those reported for the experimental control lambs (table 1). Especially interesting is the duration of the PR interval. It is identical with the PR interval of experimental control lambs, which would appear to establish the significance of the increase in the PR interval occurring in vitamin E-deficient lambs.

The increment of the PR intervals found in the E-deficient lambs reported in the present work was in agreement with the results of Gullickson and Calverley[3] who observed that 1 cow fed a vitamin E-deficient ration for one and one-half years showed an increase in the length of this interval. However, this change was not evident until the cow had been on the deficient diet for approximately seventeen months, whereas in the lambs covered by the present study, this change occurred after only eighty-one days.

In general, the changes in the character and form of the various ECG deflections which were observed are essentially in agreement with the findings of Bragdon and Levine[5] in rabbits. The evidence of disorganization of the cardiac impulse (fig. 1 C) seen in lambs 14 and 39 is reminiscent of the observations reported by Gullickson and Calverley.[3]

* Standard error.

** This is the systolic index or systole-cycle ratio calculated according to the formula devised by Bazett which is as follows:

$$K = \frac{QT}{\sqrt{RR}}$$

where QT is the time interval in seconds from the beginning of QRS to the end of the T wave and RR is the time interval occupied by a complete cardiac cycle. Any variation in K represents an alteration in the relationship of QT to the entire cycle, whereas measurements of the QT interval might reveal no actual change.

The abnormal findings in either the macro- and micropathological study [2] or the chemical analyses of the heart muscle [2] of vitamin E-deficient lambs which died suddenly could not be differentiated from those that became progressively ill. It was interesting to note that in some cases even though hearts appeared seriously injured by the deficiency of vitamin E, the ECG's of the lambs did not show any abnormality. For example, the ECG of lamb 12, taken twenty days before the death of the animal, did not differ at all from the ECG of control lambs, but the microscopic examination showed extensive degeneration of the cardiac muscle.[2]

It may be possible that the pattern of distribution of the lesions throughout the cardiac muscle might afford an explanation for these differences. This supposition may be rendered tenable when the distribution of the heart lesions in lambs showing sudden ECG changes are compared to those of E-deficient lambs not showing abnormal ECG's. It was observed that the necrotic lesions of the former were localized in a defined area, whereas those of the latter were evenly distributed and covered all the ventricular wall.

SUMMARY

The influence of vitamin E deficiency on the electrocardiogram of a group of 20 lambs has been studied. In the majority of the vitamin E-deficient lambs, the length of the PR interval increased from a normal of 0.08 second to 0.10 second and in some instances to 0.12 second. Five of the experimental lambs showed abrupt and striking electrocardiographic changes twenty-four hours before death.

References

[1] Culik, R., Bacigalupo, F. A., Thorp, F., Jr., Luecke, R. W., and Nelson, R. H.: Vitamin E. Deficiency in the Lamb. J. Anim. Sci., *10*, (1951): 1006-1016.

[2] Bacigalupo, F. A.: Unpublished data.

[3] Gullickson, T. W., and Calverley, C. A.: Cardiac Failure in Cattle on Vitamin E-Free Rations as Revealed by Electrocardiograms. Science, *104*, (1946): 312-313.

[4] Gatz, A. J., and Houchin, O. B.: Studies on the Heart of E-Deficient Rabbits. Anat. Rec., *99*, (1947): 578.

[5] Bragdon, J. H., and Levine, H. D.: Myocarditis in Vitamin E-Deficient Rabbits. Am. J. Path., *25*, (1949): 265-271.

[6] Filer, L. J., Jr., Rumery, R. E., Yu, P. N. G., and Mason, K. E.: Studies on Vitamin E-Deficiency in the Monkey. Ann. New York Acad. Sci., *52*, (1949): 284-291.

[7] Luecke, R. W., Culik, R., Thorp, F., Jr., Blakeslee, L. H., and Nelson, R. H.: Riboflavin Deficiency in the Lamb. J. Anim. Sci., *9*, (1950): 420-425.

[8] Pardee, H. E. B.: Clinical Aspects of the Electrocardiogram. 3rd ed. Paul B. Hoeber, Inc., N. Y., 1941.

The Morphogenesis of the Adrenal Glands of Chicken Embryos

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PREPARATION of this manuscript was stimulated by a review of a recent and excellent reference textbook on the adrenal gland by Hartman and Brownell.[8] In chapter 5 of the text, reference is made to the work of just two authors that deals directly with the morphogenesis of the adrenal glands of birds.

According to various investigators, the cortical substance of the adrenal gland may be derived from several possible sources. The theory of mesonephric origin is supported by Semon[28] and Hoffman.[11] Janosik,[13, 14] Fusari,[4] and Loisel[17] found a very intimate relationship between the adrenals and the gonads, and believed that the cortical portion was derived from the germinal epithelium. Valenti[28] and Soulie[26] supported the theory that the cortical substance of the adrenal develops from the peritoneal epithelium. Hays[9] stated that the anlagen which give rise to the cortical substance appear as groups of cells which migrate dorsally from the peritoneal epithelium. This worker also found that the vessels of the arterial system are almost entirely in the cortical substance.

Several theories also have been proposed to account for the development of the medullary or "chromaffin substance" of the adrenal glands. Janosik[13, 14] and Valenti[28] believed that it is derived from the germinal epithelium. Fusari,[4] Rabl,[22] Minervini,[20] Loisel,[17] Soulie,[26] and Poll[21] believed that the chromaffin substance originates from the sympathetic nervous system. Hays[9] reported that the chromaffin substance is derived from indifferent cells which wander in from the anlagen of the prevertebral sympathetic plexuses. This investigator also found that the chromaffin tissue lies in contact with the venous blood vessels. Brauer[2, 3] observed that differentiation of chromaffin cells commenced from indifferent cell aggregates about the eighth day of embryonic development and after which time these rudiments became innervated. This author also observed an early differentiation of sympathoblasts within the cell masses to the cortical cords. Processes of the sympathoblasts eventually united all of the sympathetic-chromaffin cell groups. The sympathetic-chromaffin cells were rather uniformly applied to the medial surface of the cortical cords.

Willier[27] found a thickening of the coelomic epithelium, known as the adrenal zone or cortical primordium, appearing on the anterior medioventral surface of the mesonephros early during the fourth day of incubation. Rau and Johnson[25] found that there are already signs of the grooves in the coelomic epithelium from which the cortical portion of the adrenal gland will arise in the Passer domesticus at a time equivalent to the 78-hour chicken embryo. Willier[27] stated that during the later portion of the fourth day, nodules of cells proliferate from the adrenal zone into the mesenchyme, lying ventral and lateral to the dorsal aorta. At the end of the fourth day of incubation, the cortical nodules are only slightly connected to the coelomic epithelium. By 113 hours of incubation, according to Soulie,[26] the cortical nodules became completely separated from the coelomic epithelium.

Willier[27] has observed that at ninety-six hours small groups of cells originating from the sympathetic nervous system are in close contact with the medial surface of the cortical primordium. According to Rau and Johnson,[25] these cells from the primary sympathetic chain eventually form the medullary or chromaffin cells of the adrenal.

Most investigators do not agree as to the exact relation of the sympathetic cells to the adrenal cortical nodules. Harris[7] found sympathetic cells in rather close contact with cortical nodules but no distinguishable intermingling of cells and nodules. Goormaghtigh[6] has reported that ganglion cells, but no chromaffin cells, are found between the cortical nodules of a chicken embryo incubated four days and sixteen hours.

Rabl[22] observed that the adrenal gland consists of a group of cortical cords, the cortical primordium, with a group of sympathetic cells located on the mediodorsal surface; this condition prevails up to the sixteenth day of incubation. According to Rabl[22] and Soulie,[26] this group of sympathetic cells, during the eighth and ninth days of incubation, constitutes the primordium of the medulla. Willier[27] has observed that to the end of the sixth day the medulla is composed largely of cells originating in the primary sympathetic trunks. After this time, the primary sympathetic cells are apparently supplemented by cells from the secondary or definitive sympathetic trunks. According to His[10] and Kuntz[15] the secondary sympathetic trunks first appear as a series of groups of neuroblasts located just medial to the ventral roots of the spinal nerves. At the same time, connections appear at varying intervals between the primary and secondary sympathetic nerve trunks. Abel[1] and Rau and Johnson[25] stated that the primary sympathetic trunk, as such, is incorporated in part in the

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secondary sympathetic chain of ganglia, and disappears.

Soulie[28] has observed that from the end of the sixth day of incubation the mass of sympathetic cells in contact with the cortical primordium shows two types of cells: large ganglionic, and small nonganglionic cells. Rabl[26] stated that these cell types are visible at least to the sixteenth day of incubation and regarded the nonganglionic cells as "embryonic ganglionic cells," while Soulie[28] called them "parasympathetic cells." According to Willier,[32] these cells penetrate between the cortical cords to become the medullary cords. Lillie[16] stated that the nonganglionic cells possess large vesicular nuclei with granular contents, and that they are "precisely like the specific cells of the ganglion, perhaps a little smaller, and without axones."

The exact time of ingrowth of the cells from the sympathetic nervous system into the adrenal gland varies according to the investigator. Fusari[4,5] found that penetration occurred at ninety-six hours of incubation. Soulie[28] observed small "parasympathetic cells" between the cortical cords in the 200-hour embryo. Lillie[16] stated that on the eighth day of incubation, strands of sympathetic medullary cells can be traced into the superficial portion of the adrenal, and by the eleventh day the cells have penetrated through one third of the adrenal. According to Rabl,[26] penetration is still in progress in the 16-day embryo. Goormaghtigh[6] described an ingrowth of chromaffin cells, beginning at six days and nine hours of incubation; these cells were of sclerotome origin.

Willier[32] has observed that the cortical substance of the adrenal gland first assumes the form of definite cords at approximately six days of incubation and at 135 hours according to Soulie.[28]

Willier[32] also observed that the laterality of the adrenal is determined at an early stage because of differences in the growth capacities of the right and left primordia. The right adrenal is usually smaller than the left. These results are in agreement with those of Goormaghtigh.[6] Sauer and Latimer[24] observed that the amount of adrenal cortex is more variable in the adult female than in the male.

Hays[9] observed that the entire venous system of the adrenal gland is derived from the subcardinal veins; within the adrenal glands, the vessels of the subcardinal system are sinusoidal in character. Miller,[19] working on the development of the postcaval vein, arrived at a similar conclusion. Hays[9] also found that there is greatest activity in the development of the vascular systems during the period of greatest influx of cells from the anlagen of the prevertebral sympathetic plexuses.

By use of the intestinal strip method, Hogben and Crew[12] have found that the adrenal gland of chicken embryos becomes functional at the sixteenth day of incubation. Lutz and Case[18] determined the mydriatic potency of the adrenal glands of 42 chicken embryos at various ages between seven and twelve days of incubation and found an active agent as early as the eighth day.

Sun[27] and Venzke[29] have reported on the weights of adrenal glands in developing chicken embryos.

Venzke[29] has attempted to correlate the morphological observations with the normal physiology of the endocrine system in the developing embryo.

MATERIALS AND METHODS

All embryos and chickens used in this study were of a pure single comb, White Leghorn stock maintained by the Department of Poultry Husbandry at Iowa State College. Throughout the study, the fertile eggs were obtained from the same group of hens.

A series of 420 embryos, ranging from twelve hours of incubation to hatching, was used for the morphological studies.

The adrenal glands were fixed in Helly's fluid and Orth's fluid, and embedded in Altmann's paraffin mixture. All sections were cut at 6 μ to 8 μ, and stained by the iron-hematoxylin method.

OBSERVATIONS

The cortical primordia of the adrenal glands make their first appearance at varying times between ninety and ninety-six hours of incubation. These cells appear as a thickening of the coelomic epithelium, dorsal to the open hind gut, ventral and medial to the anterior pole of the mesonephros, ventral and somewhat lateral to the subcardinal veins, ventral to the aorta, and medial to the anterior end of the germinal ridge. As these cells develop, they become larger and more rounded in shape than the small flat coelomic or peritoneal cells from which they are derived. The nuclei of the cortical cells also differ from those of the parent peritoneal epithelial cells in that they stain less deeply because of the scanty amount of chromatin material. The cytoplasm of the cortical cells also stain less deeply than that of the peritoneal cells.

Mitotic figures are seen in many of the cell nuclei. As a result of this marked mitotic activity, the cortical cells at 110 hours of development have increased greatly in number. The cortical cells have been pushed dorsally and grouped into a solid body on either side of the base of the mesentery, slightly medial and ventral to the anterior pole of the mesonephros. This moderate change in position of the cortical tissue is the result of the increased rate of growth of the mesonephros, body wall, and mesentery. From the solid mass of cells ly-

ing on the peritoneal epithelium, a chain of cells can be traced dorsally toward the ventral level of the aorta. These cells lie in the mesenchyme between the mesonephros and the aorta. Many also migrate laterally to the subcardinal vein, surrounding it ventrally and medially. The character of the cortical cells has not changed in regard to their staining properties.

On each side of the dorsal surface of the aorta, there is a cord of nerve cells known as the primary sympathetic nerve trunk. This trunk shows a slight ganglionic enlargement opposite each spinal ganglion.

At 120 hours of development, the cortical cells have become detached from the peritoneal mesothelium and have migrated dorsally (fig. 1). They appear as scattered groups of cells extending from the dorsal surface of the subcardinal veins dorsally to a level midway along the lateral wall of the aorta. Approximately the same level is reached on both sides of the aorta. This locates the cortical cell groups scattered through the mesenchyme between the medial side of the mesonephros and the aorta. Almost the entire region has been invaded by the migrating cortical cell groups. The posterior limits of the developing cortical tissue lies at a point where the subcardinal veins anastomose in the median plane ventral to the dorsal aorta.

There are a few erythrocytes scattered throughout the cortical cells. There is no connection between the cortical cells and the subcardinal veins; however, the vein does push dorsally for a short distance into the cortical cells at several points.

At this time, small branches pass into the cortical tissue from the anterior mesonephric arteries.

At 120 hours of incubation, large oval cells are observed migrating ventrally from the primary sympathetic trunks on either side of the dorsal aorta. These were present in very small numbers in the 110-hour embryo. They usually migrate singly, but occasionally they are found in pairs. As these sympathetic cells approach the loose cortical cell groups, they migrate between the cortical tissue and the dorsal aorta. At this time, there is no intimate association of the two kinds of cells. The sympathetic cells are characterized by their large size and deep staining.

From 120 to 134 hours of development, there has been a noticeable increase in the amount of cortical tissue. The cortical cells are no longer scattered throughout the mesenchyme between the aorta and the mesonephros but have become aggregated into large groups of cells. These large, solid groups of cells lie dorsomedially to the subcardinal veins and ventral to the mesonephric arteries which usually cross over the anterior extremities of the cell groups.

At 134 hours of development, some of the cells migrating from the primary sympathetic trunks enter the cortical tissue, or attach themselves to the surface of cortical cell groups. Some of the sympathetic cells have migrated deeply into the cortical primordium, since the cells of the cortical tissue are not compact.

After 148 hours of development, the cortical cells have become closely grouped into an ovoid mass on each side of the dorsal aorta. The cell nuclei are beginning to stain more deeply because of increased granulation of the cytoplasm. The cell outlines are quite irregular.

Small strands of mesenchymal tissue are found between groups of cells. The adrenal is separated from the mesonephros by a thin layer of flattened mesenchymal cells. The venous circulation is increasing.

Sympathetic cells continue to invade the cortical tissue. There is a group of sympathetic cells on the dorsomedial surface of the anterior pole of each gland. This mass of sympathetic cells in contact with the gland shows two kinds of cells: large ganglionic and small nonganglionic cells. The nonganglionic cells are more numerous next to the periphery of the gland. However, at this time both types of cells are migrating into the gland. These cells arise from the secondary or definitive sympathetic trunks. Prior to this time, the sympathetic cells were derived from cells originating in the primary sympathetic nerve trunks.

The ganglionic cells are identified by their large size, irregular outline, large nucleus, and amount of cytoplasm. The nonganglionic cells are smaller, round in outline, and possess a small nucleus and little cytoplasm.

After 165 hours of development, the mass of cortical tissue has increased (fig. 2). The cells are arranging themselves in chains, and sinusoids are appearing between them. The nuclei of the cortical

cells contain more chromatin material than previously, and they stain more deeply. Mitotic figures are present in large numbers. The mesonephros has increased considerably in size and has pushed ventrally into the abdominal cavity. As a result, the adrenal gland contacts the mesonephros only on its dorsomedial surface. Connective tissue fibers are gathering around the gland and will eventually form a tissue capsule. A few of these fibers extend into the parenchyma between the cells from the surface of the gland.

The glands have increased in size to the point that they lie ventrally and medially to the posterior cardinal veins. A branch enters this vsesel from each gland, and leaves the gland on its dorsolateral surface. Blood appears in the sinusoidal spaces between the cell cords.

An artery is given off by each of the anterior mesonephric arteries. These arteries travel anteriorly, one along the lateral border of each gland. They give off two branches to each gland, one near the posterior and one near the anterior poles of the gland. These then branch on the surface of the gland and finally enter it as several small branches. Another small artery extends directly from the aorta to the posterior pole of the left adrenal. This artery branches on the surface of the gland and enters the parenchyma. These arterial branches continue to divide into capillaries within the parenchyma.

The sympathetic cells at 165 hours are showing slight differential changes. It is now difficult to distinguish them from the cortical cells. However, with the iron-hematoxylin staining method, the sympathetic cells take a deeper blue color than the cortical cells. The nuclei of the sympathetic cells have become more granular, whereas previously they were clear. The invading cells have a slight tendency to arrange themselves in cords, although many individual cells are present. The mesenchyme surrounding the gland contains large numbers of these cells, indicating an increase in the migration of cells from the sympathetic trunks. The cells enter the glands from all surfaces.

The adrenal glands, after 177 hours, have about the same cross sectional diameter as the dorsal aorta, approximately 150 μ. More connective tissue is penetrating the cortical cells. Development of the venous sinusoids continues steadily, and the arterial capillary network is becoming more complex within the parenchyma.

At 177 hours, the mesenchyme surrounding the adrenal glands is packed with cells from the sympathetic trunks. These cells are entering the gland on all sides, but especially on its dorsal and medial surfaces. This apparently is the peak of migration of cells from the sympathetic trunks.

After 191 hours, the adrenal glands are approximately 250 μ in diameter through the center, and 1.5 mm. in length. There is an increase in the number of chromaffin cells which are arranged within the adrenal parenchyma in cords. Large numbers of them are grouped around venous vessels, and some are scattered individually throughout the cortical cells.

At 206 hours, three tvpes of cells comprise the medullary substance of the glands. The chromaffin cells, easily distinguished by their darker staining, possess fine, dark staining granules in their

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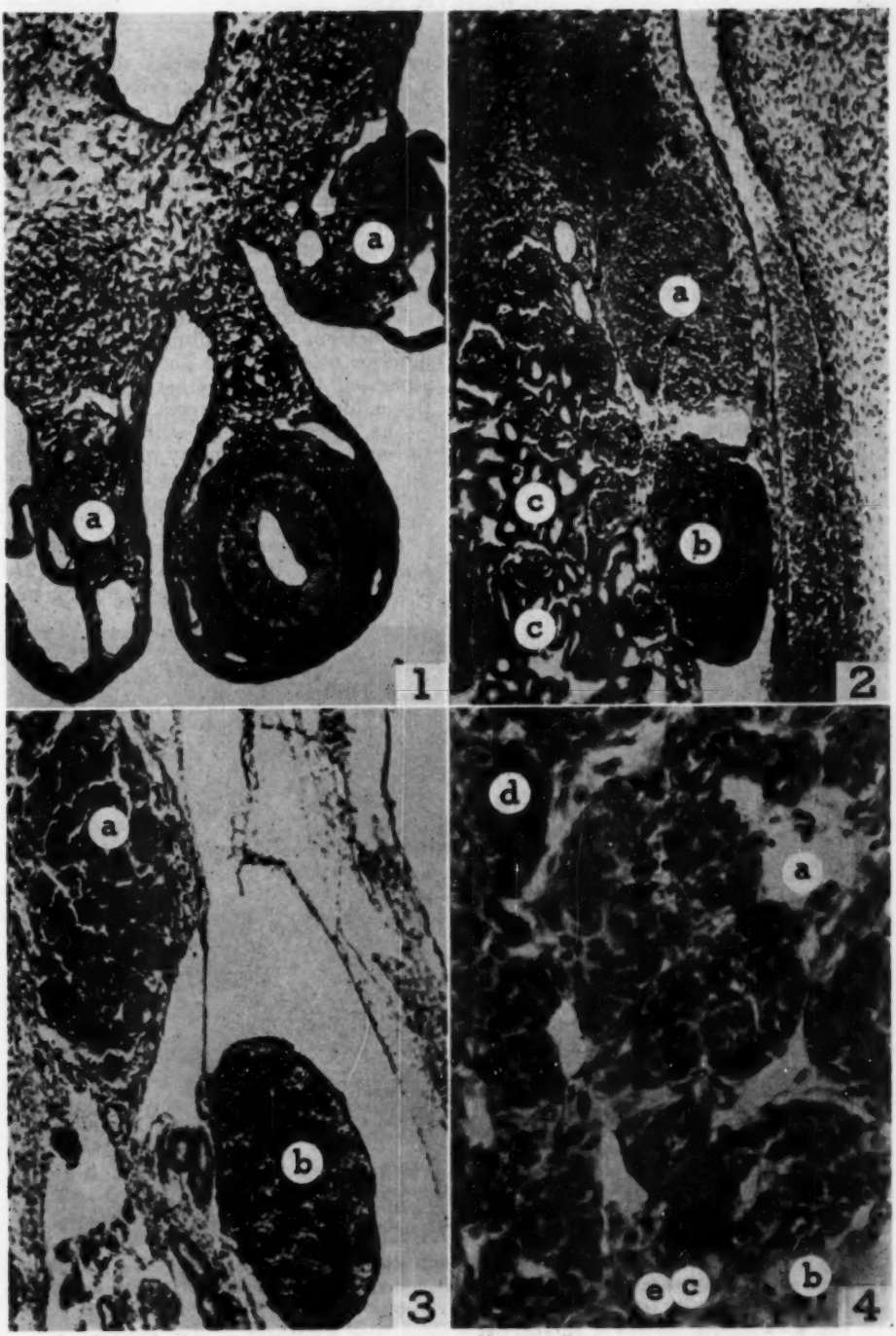
Fig. 1—A transverse section taken through the abdominal cavity of a 120-hour chicken embryo showing a portion of the adrenal cortical primordium. (a) Adrenal cortical primordium. Hematoxylin-eosin. x 200.

Fig. 2—A sagittal section taken from a 165-hour chicken embryo presenting the relations of the gonad, mesonephros, and adrenal gland. (a) Adrenal gland; (b) gonad; (c) mesonephros. Hematoxylin-eosin. x 100.

Fig. 3—A sagittal section taken from a 228-hour chicken embryo presenting the cordlike arrangement of the adrenal tissue and its relation to the testis. (a) Adrenal gland; (b) testis. Hematoxylin-eosin. x 100.

Fig. 4—A sagittal section of the adrenal tissue taken from a 272-hour chicken embryo. (a) Venous sinusoid; (b) large ganglionic cell; (c) cortical cord; (d) nonganglionic cell; (e) chromaffin cells. Iron hematoxylin. x 400.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cytoplasm. These cells are grouped in some cases while, in other instances, they form strands. They are closely apposed to the cortical cell cords; in some instances, they form an intimate part of them. The most common cell is the small nonganglionic type, migrating in from the ganglionic mass along the dorsomedial surface of the gland. These cells migrate into the gland with nerve fibers. The large ganglion cells are confined primarily to the periphery of the adrenal; occasionally one is found deep in the gland. The nonganglionic cells occur in groups, while the ganglionic cells occur singly in intimate association with the nonganglionic cells.

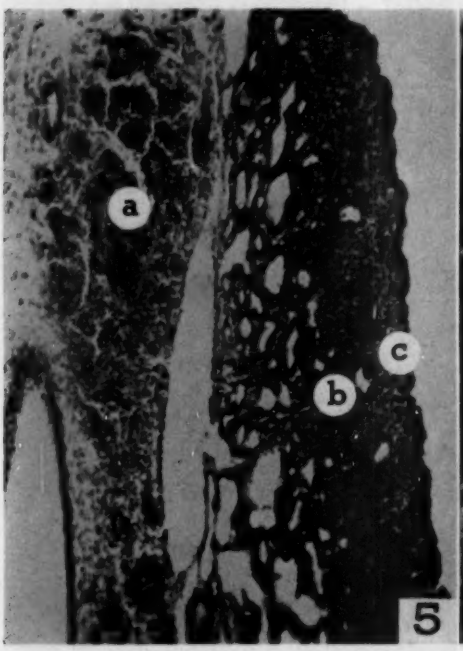
After 213 hours, the cortical cells are arranged in cords without distinct cellular outlines. This gives the cell cords a syncytial appearance. Mitotic figures are frequently observed.

The diameter of each gland, at 228 hours, is approximately 300 μ; the length, 1.75 mm. (fig. 3). Fewer mitotic figures are present in the cell nuclei; this indicates a decrease in the rate of growth. Most of the gland is composed of cortical tissue. Venous vessels are surrounded, on at least one surface, by chromaffin cells whose tissue seems to have no regular arrangement. Chromaffin and nonganglionic cells are about equally divided in the gland, with a slight preponderance of the nonganglionic type. All three types of cells are found in the ganglionic masses along the glands.

The left adrenal is covered by the left ovary except for a small portion of its most anterior pole (fig. 5). The two glands are separated from each other by connective tissue, blood vessels, nerves, and ganglia.

The venous sinusoids still open into the subcardinal veins in many places. No definite vessels carry the blood from the sinusoids to the subcardinal veins. The arterial supply has not changed.

There is a noticeable increase in the size and extent of the venous sinusoids at 240 hours of development. The glandular sub-



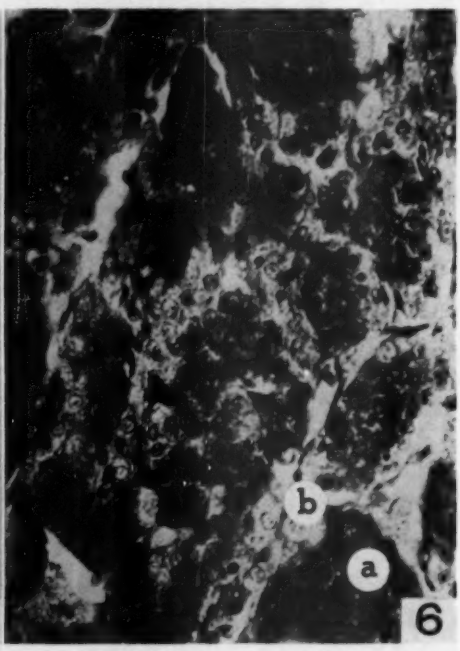


Fig. 5—A sagittal section taken from a 284-hour chicken embryo showing the relation of the adrenal and the left ovary. (a) Adrenal gland; (b) ovarian medulla; (c) ovarian cortex. Hematoxylin-eosin. x 100.

Fig. 6—A sagittal section of adrenal tissue taken from a 400-hour chicken embryo. (a) Cortical cord; (b) chromaffin cells. Iron hematoxylin. x 400.

stance is irregularly spaced by venous sinusoids which form a network throughout the entire gland. The cortical cells are arranged in irregular cords, and form the foundation for the chromaffin tissue. Blood vessels and sinusoids pass between the cell cords.

The adrenal glands, after 252 hours of development, have increased in size so that they completely occupy the space between the anterior pole of the kidney and aorta on the right and left sides. The adrenal glands are so closely attached to the kidney that the two structures appear to be continuous. In reality, they are separated by at least a mesothelial layer. Sympathetic ganglionic masses, which have been situated on the dorsomedial surface of the adrenal since 191 hours, are found between the aorta and adrenal. They are gradually increasing in size.

The chromaffin cells of the adrenal are becoming very granular, and their nuclei are vesicular, as compared with the more granular nuclei of the cortical cells. The positive chrome reaction, which these cells have recently acquired, differentiates them from the surrounding ganglionic cells. However, occasionally a large ganglion cell is found, deep in the parenchyma, which does not take this stain. Nonganglionic cells are less prevalent than chromaffin cells in the gland. The ganglionic masses contain all three types of cells, with the nonganglionic predominating.

At 272 hours, the cortical cell cytoplasm shows more granulation. Ganglionic cells extend from the periphery into the gland between the cords of cortical cells (fig. 4). Occasionally, an isolated ganglionic cell is present in the parenchyma, together with nonganglionic cells. The ganglionic masses are composed of three types of cells with the nonganglionic cells predominating. More, large, ganglionic cells are found in the glands. The nonganglionic cells are more numerous than the chromaffin cells. The connective tissue fibers between the cortical cells are increasing in number, and the connective tissue capsule is thickening.

After 284 hours, the adrenal glands are increasing more rapidly in diameter than in length. They now measure 1.5 mm. in length, and 0.4 mm. in diameter. The connective tissue capsule has increased to 5 μ.

The cortical cells of the adrenal glands, after 295 hours of development, have rather distinct outlines and granular cytoplasm. They vary in shape from round to hexagonal.

The outlines of the chromaffin cells are not distinct; some are elongated while others are almost round. The granulation of the cytoplasm in these cells is very marked, causing the cells to take a definite chrome reaction.

At 307 hours, the two veins carrying the venous blood from the adrenals to the subcardinal veins are completely surrounded by chromaffin and ganglion cells.

Growth of the glands is the only change which has occurred between 307 and 331 hours of development.

After 331 hours, the ovary of the chick is in very close proximity to the adrenal; the two glands are separated by a thin band of plethoric connective tissue (fig. 5). After 357 hours, the glands measure approximately 0.6 mm. in diameter and 1.5 mm. in length.

At 388 hours, the adrenal glands are more compact, and the venous sinusoids are less voluminous. The cytoplasm of the cortical cells is very dense and granular and seems to have reached a peak in granulation. Migration of nonganglionic and ganglionic cells from the ganglionic masses with sympathetic fibers and blood vessels continues. The ganglionic cells within the glands are usually surrounded by nonganglionic and chromaffin cells.

To some degree, the chromaffin cells resemble the nonganglionic cells. Their nuclei are approximately the same size and structure; however, the chromaffin cells possess more highly granular cytoplasm. The nonganglionic cells are more numerous than ganglionic cells within the gland.

After 400 hours, the chromaffin tissue is better developed than at any previous stage (fig. 6). Histologically, the cortical and chromaffin cell cords are differentiated by their structure and staining reaction. The bulk of the gland consists of a network of cortical cords, with chromaffin cords and blood vessels lying between them. Generally, the central portion of the adrenal is distinctly sinusoidal in character, while the surrounding periphery is quite compact.

Migration of the sympathetic cells from the ganglionic masses does not occur at this time. Masses of ganglionic, nonganglionic, or both cell types together are found contiguous with the surface of the adrenals.

These two cell types usually appear together in irregular groups of variable size. Either cell type may be the more common in the group. The sympathetic nerve fibers are becoming extensively distributed throughout the adrenal substance.

At 438 hours, many of the glands studied showed the chromaffin tissue confined to the periphery. The large veins leaving the gland were completely surrounded by chromaffin tissue. The ganglionic masses contiguous with the surface of the gland contain all three types of cells but a preponderance of chromaffin cells are present. Very few ganglionic or nonganglionic cells were found in the adrenal substance.

The connective tissue capsule is increasing about the glands and between the cortical cords. At 450 hours, the adrenal capsule varies in thickness from 10 to 25 μ.

At 474 hours, there occurs an increased migration of nonganglionic and ganglionic cells from the ganglionic masses into the adrenal substance.

At 498 hours, contiguous with the surface of the adrenals are ganglionic masses. Some of the masses are composed of three types of cells; ganglionic-nonganglionic cells predominate in these masses. There are masses composed entirely of ganglionic cells, but they are small in size and few in number.

From these masses, sympathetic nerve fibers are seen penetrating deeply into the gland substance. Along their course are found all three types of cells. Chromaffin and nonganglionic cells are the most common along the course of these nerve fibers.

A rather dense connective tissue capsule surrounds the glands. From the capsule, fine connective tissue fibers are detached between the cords of parenchymatous cells.

Much of the chromaffin tissue is confined to cords of cells near the periphery of the glands. These cords are separated from each other by fine connective tissue fibers and mesothelial cells. Between these cords, there is a fine network of capillaries and small sinusoids. However, the chromaffin tissue is distributed throughout the glands and some portion of the chromaffin cell cord is always in contact with a venous sinusoid.

The cortical cords are irregularly distributed throughout the gland and are separated from one another and from the chromaffin cords by fine connective tissue fibers.

The central portion of the glands is highly excavated by the venous sinusoids. These sinusoids are very small near the periphery of the glands.

DISCUSSION

The cells of the cortical primordia of the adrenal gland first appear between ninety and ninety-six hours of incubation. This observation has previously been made by Hays [9] and Willier.[32] These cells are derived from the medioventral coelomic epithelium over the anterior pole of the mesonephros. This observation is in agreement with that of Soulie [26] and Hays.[9]

Between 110 and 120 hours of incubation, large oval cells migrate ventrally from the primary sympathetic trunks on either side of the dorsal aorta. These sympathetic cells, as they approach the cortical cells, migrate between them and are found deeply in the cortical tissue at 134 hours. They give rise to the chromaffin tissue in the adrenal glands. This observation substitutes the work of the majority of investigators.[5, 9, 23, 26, 32] Those investigators not in agreement have been cited in the literature review.

After 148 hours, masses of sympathetic cells appear along the dorsomedial surfaces of the adrenal glands. These sympathetic cell masses consist of two types of cells, ganglionic and nonganglionic cells, which arise from the secondary or definitive sympathetic trunks. Prior to this time, the sympathetic cells are derived from the primary sympathetic nerve trunks. Both of these cell types migrate into the adrenal tissue at 165 hours.

Most investigators are of the opinion that the nonganglionic cells are of ectodermal, rather than mesodermal, origin. The present study agrees with this observation. However, in this study no intermediate cell stages are found between the ganglionic and nonganglionic cells developing into chromaffin tissue. So far as the author is aware, no description of such cellular transformation occurs in the literature. It is interesting to note that the chromaffin cells resemble the nonganglionic cells, except that they possess more granular cytoplasm. Perhaps the nonganglionic cells differentiate into chromaffin cells af-

ter they migrate into the adrenal tissue. The nonganglionic cells are more numerous than the ganglionic cells, which is also indicative that they may change into chromaffin tissue. However, there seems to be more indirect evidence to substantiate the hypothesis that the ganglionic cells produce chromaffin tissue than that the nonganglionic cells produce it. As development of the adrenal gland progresses, the number of ganglionic cells decreases within the parenchyma. These cells are usually confined to the periphery of the gland. Near the close of the incubation period, the greatest portion of chromaffin tissue is also confined to the periphery of the gland (fig. 6). Therefore, it is assumed that because the ganglionic cells decrease in number within the gland and are confined to the periphery and, because most of the chromaffin tissue is located in this area, the ganglionic cells are responsible for chromaffin cell formation.

Willier [32] also obtained evidence that supports the above hypothesis. Willier found that "a suprarenal forms in the graft from a Wolffian body isolated from a 4-day embryo, two days before the secondary sympathetic nerve trunks begin to form." From the observations made in this study the nonganglionic cells are not distinguishable until 148 hours. Therefore, it may be assumed that the ganglionic cells are transformed into chromaffin tissue. Willier, however, points out that the secondary sympathetic cells may contribute indirectly to the formation of the medullary cords. Poll [21] thought that cells from the primary sympathetic system are responsible for innervation to the adrenal glands. Goormaghtigh [6] considered these cells essential for nerve supply and for the production of chromaffin tissue.

Therefore, the exact origin of the chromaffin cells is still unknown. There is evidence that both the ganglionic and nonganglionic cells may be transformed into chromaffin cells. If the ganglionic cells are the sole source of chromaffin cells, then perhaps the nonganglionic cells serve as supporting cells. Perhaps the medulla is composed of chromaffin cells which have nervous connections and are essential in the production of epinephrine, and cells which are not. In this case, it may be plausable to assume that the ganglionic cells give rise to the chromaffin cells which produce epinephrine while the nonganglionic cells produce chromaffin tissue which is not secretory in nature. However, in this connection two types of chromaffin cells can not be distinguished. Furthermore, Sun [27] has found that, as the chromaffin tissue increases, so also the epinephrine content of the glands increases.

The time of penetration of the sympathetic cells into the adrenal glands varies according to the investigator. Fusari [4] found sympathetic cells among the cortical tissue at the end of the fourth day. Rabl [22] and Soulie [26] observed sympathetic cells, contiguous with the cortical primordium, at the end of the fourth day. From the sixth to the ninth days, these investigators observed that the sympathetic cells formed a ganglionic mass on the mediodorsal face of the cortical tissue. From this ganglionic mass, sympathetic medullary cells began to penetrate the cortical cords on or about the eighth day. Willier [32] is of the opinion that in a graft from a 4-day embryo, "the primary sympathetic cells contiguous with the cortical primordium multiply to form a mass before any cells separate and penetrate as medullary cells between the cortical cords." The present study indicates that sympathetic cells migrate into the adrenal glands from 110 hours until hatching. It is evident from this observation that the migration of sympathetic cells into glands occurs in waves throughout the incubation period. During the early stages of development, the sympathetic cells migrate between the loosely arranged cortical cords. After a definite capsule is formed, the cells migrate into the gland along nerve fibers. Rabl [22] stated that penetration of sympathetic cells is still evident in a 16-day embryo. Wiesel,[31] working with human material, found sympathetic cells migrating into the glands until at least the tenth year.

The relationship between chromaffin and cortical cells is interesting. The chromaffin cells are found between the cortical cords and are associated with the sympathetic cells which are found within the gland or contiguous with the surface of the gland. The sympathetic cells are not found in contact with the cortical cells but only with chromaffin cells and connective tissue. Connective tissue separates the chromaffin and

sympathetic cells from the cortical cells. Furthermore, chromaffin cells are always in contact with a venous sinusoid. These relationships suggest that the sympathetic cells differentiate into chromaffin cells. Just what influence stimulates the change of sympathetic cells into chromaffin cells is unknown. From casual observation, one might assume that the cortical cells stimulate the transformation of cells into chromaffin tissue. However, this does not explain the production of chromaffin cells outside of the adrenal glands. As noted above, masses of ganglionic tissue containing ganglionic, nonganglionic, and chromaffin cells are located outside the adrenal glands during the developmental period and at hatching time. These ganglionic masses simulate paraganglia.

A morphological study of the developing adrenal glands does not answer the question as to what role the sympathetic cells play in the differentiation of the cortical tissue. There is no evidence that the differentiation of cortical tissue is dependent on sympathetic cells.

SUMMARY

The morphogenesis of the adrenal glands of 420 developing chicken embryos was studied by means of serial, sagittal, transverse, and frontal sections taken at approximately twelve-hour intervals from the twelfth hour of incubation until hatching.

The anlagen which gives rise to the cortical substance of the adrenal appear between ninety and ninety-six hours as groups of cells derived from the coelomic epithelium.

Sympathetic cells derived from the primary sympathetic trunks have migrated between the cortical cells in the 134-hour embryo.

Masses of sympathetic cells appear along the dorsomedial surfaces of the adrenal glands at 148 hours. These sympathetic cell masses consist of two types of cells, namely ganglionic and nonganglionic. It is believed that these cells arise from the secondary or definitive sympathetic trunks. Both of these cell types migrate into the adrenal tissue at 165 hours.

Indirect evidence indicates that the sympathetic cells, which arise from both the primary and secondary sympathetic trunks, transform into chromaffin tissue, since the migration of sympathetic cells into the adrenals takes place continuously throughout the incubation period. Because the penetration of sympathetic cells is more pronounced at certain periods, it is assumed that they migrate in waves.

The origin of chromaffin cells from the sympathetic cells is suggested by the scarcity of nonganglionic and ganglionic cells in the adrenal glands at certain stages during development, indicating an almost complete transformation of sympathetic cells into chromaffin cells. The intimate association of sympathetic and chromaffin cells is also indicative of sympathetic cell transformation.

Some indirect evidence suggests that the chromaffin cells may also be derived from nonganglionic cells, since they resemble nonganglionic cells, and because nonganglionic cells are always numerous next to the cortical cords, where chromaffin cells differentiate.

The differentiation of sympathetic cells into chromaffin cells seems to be independent of the influence of cortical cords. This is indicated by the fact that masses of sympathetic cells not in close proximity to the cortical cords differentiate into chromaffin cells.

The chromaffin cords arrange themselves within the gland tissue so that some portion is next to a venous sinusoid. The arterial system is confined almost entirely to the cortical tissue.

References

[1] Abel, W.: Further Observations on the Development of the Sympathetic Nervous System in the Chick. J. Anat. and Physiol., *47*, (1912): 35-72.

[2] Brauer, A.: A Topographical and Cytological Study of the Sympathetic Nerve Components of the Suprarenal of the Chick Embryo. Anat. Rec., *51*, (1931): 61.

[3] Brauer, A.: The Differentiation of the Sympathetic Nervous Components of the Chick Suprarenal in Chorioallantoic Grafts. Anat. Rec., *60*, (1934): 60-61.

[4] Fusari, R.: Contribuzone allo studio dello sviluppo delle capsule surrenali e del simpatico nel pollo e nei manniferi. Arch. Sci. Med., *16*, (1892): 249-301.

[5] Fusari, R.: Contribution a l'étude du développment des capsules surrénali et du sympathique chez le poulet et les mammifères. Arch. ital. de biol., *18*, (1893): 161-182.

[6] Goormaghtigh, N.: Organogenèse et histogenèse de capsule surrénale et du plexus coelinque. Arch. di Biol., *31*, (1921): 83-172.

[7] Harris, H. A.: Observations on the Origin of the Adrenal Glands in the Chick. Masters' Thesis, University of Chicago. 1928.

[8] Hartman, F. A., and Brownell, K. A.: The Adrenal Gland. Lea & Febiger. Philadelphia, (1949): 74.

[9] Hays, V. J.: The Development of the Adrenal Glands of Birds. Anat. Rec., *8*, (1914): 451-474.

[10] His, W., Jr.: Über die Entwickelung des Bauchsympathicus beim Hühnchen und Menschen. Arch. f. Anat. u. Physiol. Suppl., *137*, (1897): 170.

[11] Hoffman, C. K.: Étude su le développment de l'apparéil uro-genital des oiseaux. Verhandel. d. k. Akad. v. Wetensch., *1*, (1893): 1-54.

[12] Hogben, L. T., and Crew, F. A. E.: Studies on Internal Secretions. II. Endocrine Activity in Foetal and Embryonic Life. Brit. J. Exptl. Biol., *1*, (1923): 1-13.

[13] Janosik, J.: Bemerkungen über die Entwicklung der Nebenniere. Arch. f. mikr. Anat., *22*, (1883): 738-746.

[14] Janosik, J.: Histologisch — Embryologische Untersuchungen über das Urogenital-system. Sitzungsb. Akad. Wissensch., Math.—naturw. Cl., Abt. 3, *91*, (1885): 97-199 and *99*, (1890): 260-288.

[15] Kuntz, A.: The Development of the Sympathetic Nervous System in Birds. J. Comp. Neurol., *20*, (1910): 283-308.

[16] Lillie, F. R.: The Development of the Chick. 2nd ed. Henry Holt and Company, New York, 1919.

[17] Loisel, G.: Les phenomènes de secretion dans les glandes genitales. Revue générale et faits nouveaux. J. de l'anat. et physiol., *40*, (1904): 536-562.

[18] Lutz, B. R., and Case, M. A.: The Beginning of Adrenal Function in the Embryo Chick. Am. J. Physiol., *73*, (1925): 670-678.

[19] Miller, A. M.: The Development of the Postcaval Veins in Birds. Am. J. Anat., *2*, (1903): 283-298.

[20] Minervini, R.: Des capsules surrénales, Développment, structure, fonctions. J. de l'anat. et physiol., *40*, (1904): 449-492, 634-667.

[21] Poll, H.: Die Entwicklung der Nebennierensysteme. (In Hertwig, O., ed. Handbuck der vergleichenden und experimentellen Entwickelungslehre der Wirbeltiere, vol. 3, pt. 1, Jena Fischer, *9*, [1906]: 443-616.)

[22] Rabl, H.: Die Entwicklung und Structur der Nebennieren bei den Vögeln. Arch. f. mikr. Anat., *38*, (1891): 492-523.

[23] Rau, S. A., and Johnson, P. H.: Observations on the Development of the Sympathetic Nervous System and Suprarenal Bodies in the Sparrow. Proc. Zoöl. Soc. London, (1923): 741-768.

[24] Sauer, F. C., and Latimer, H. B.: Sex Differences in the Proportion of the Cortex and the Medulla in the Chicken Suprarenal. Anat. Rec., *50*, (1931): 289-298.

[25] Semon, R.: Die indifferente Anlage der Keimdrüsen beim Hühnschen und ihre Differenzierung zum Hoden. Jena. Zeit. Naturwiss., *21*, (1887): 46-86.

[26] Soulié, A. H.: Recherches sur le developpement des capsules surrénales chez le vertébrés superieurs. J. de l'anat. et physiol., *39*, (1903): 197-293.

[27] Sun, T. P.: Histo-Physiogenesis of the Glands of Internal Secretion—Thyroid, Adrenal, Parathyroid, and Thymus of the Chicken Embryo. Physiol. Zoöl., *5*, (1932): 384-396.

[28] Valenti, G.: Delle capsule surrenali nel pollo ed in alcuni mammiferi. Pisa Soc. Tosc. Atti (Mem.), *10*, (1889): 122-148.

[29] Venzke, W. G.: Endocrine Gland Weights of Chicken Embryos. Growth, *7*, (1943): 265-271.

[30] Venzke, W. G.: The Endocrine Gland System. Morphology and Physiology Correlated During Development. Vet. Student, *5*, (1942): 61-63, 94-98.

[31] Wiesel, J.: Beiträge zur Anatomie und Entwickelung der menschlichen Nebenniere. Anat. Hefte., *19*, (1902): 481-522.

[32] Willier, B. H.: A Study of the Origin and Differentiation of the Suprarenal Gland in the Chick Embryo by Chorioallantoic Grafting. Physiol. Zoöl., *3*, (1930): 201-225.

Gross and Microscopic Anatomy of the Respiratory System of the Turkey

II. The Larynx, Trachea, Syrinx, Bronchi, and Lungs

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Literature Cited

The gross anatomy of the chicken's larynx has been described by many authors, including Bradley,[5] Foust,[11] Sisson and Grossman,[17] Ellenberger and Baum,[10] McLeod and Wagers,[15] and Kaupp.[14] All reports agreed that the chicken has no thyroid cartilages, vocal cords, or epiglottis.

The gross anatomy of the chicken's trachea was described by McLeod and Wagers.[15] They stated that the rings were complete but did not indicate that they overlapped. Bradley stated that the tracheal rings of the chicken were of alternate sizes. He also stated that the tracheal wall was composed of four layers: The outer areolar connective tissue, the cartilaginous rings, the submucosa, and the epithelium. Batt[1] described three layers in the tracheal wall of the chicken.

Juillet[13] made a rather complete report on the histology of the bronchi and lungs of birds. He observed that the wall of the extrapulmonary bronchus consisted of a mucous layer, cartilaginous rings, and a thin, loose connective tissue layer covered by epithelium from the interclavicular air sac which surrounds it. Both Kaupp[14] and McLeod and Wagers[15] reported on the relation of the gross structure of the chicken's syrinx to its voice-producing qualities. Both reports noted the specialization of the last tracheal and first bronchial rings but did not give the syrinx precise anatomical demarcation.

McLeod and Wagers,[15] Bradley,[5] Kaupp,[14] and Sappey[16] all agreed that the primary bronchus in the chicken widened just after its entrance into the lung, forming a vestibulum. They disagreed regarding the arrangement and anastomoses of the respiratory tubules. Kaupp[14] stated that they anastomosed and had dilated terminations. Anastomoses between terminal tubules were suggested by Sappey.[16] McLeod and Wagers[15] by their description and drawing indicated that the respiratory tubules anastomosed with each adjacent tubule. However, Hazelhoff[12] clearly portrayed, in a drawing of a parabronchus, that the terminal tubules are finger-like projections of different lengths which arise from the parabronchus at right angles. Juillet[13] was one of the first to point out the principal differences between mammalian and avian lung structure. His manuscript referred to many of the earliest works on avian anatomy.

Dotterweich[7-9] described the location and direction of the secondary bronchi within the lungs. This was done by injecting lampblack into the air sacs of birds. Vos[18] continued this work by having live ducks breathe air laden with carbon particles. Hazelhoff[12] injected powdered charcoal into the air sacs of crows, pigeons, chickens, and herons in order to study the aerodynamics of the lungs.

Materials and Methods

The pharynx has not been investigated separately. The most complete and recent investigation on the pharynx of the chicken was made by Calhoun.[4] In making sections of the larynx, small sections of the adjacent parts of the pharynx were attached and consequently processed.

The larynges of 17 turkeys were fixed for twenty-four hours in Zenker's fluid or formalin, embedded in paraffin, and sectioned. Spaced serial sections were made from the larynx of 2 birds, so that any abrupt change in epithelium or basic structure would be detected. One set of spaced serial sections was stained by the Tänzer-Unna orcein method in order to identify the amount and distribution of the elastic tissue which was present. Sections of larynx were also stained by the Masson method, and still another set of sections was stained by Heidenhain's "azan" procedure. Three fresh and seven formalin-fixed specimens of the larynx were studied grossly.

The tracheas from 11 turkeys were processed in the usual manner following fixation in 10 per cent formalin or Zenker's fluid. They were not decalcified. The following stains were used: hematoxylin and eosin, Heidenhain's "azan," Mayer's mucicarmine, Tänzer-Unna's orcein, and Kornhauser's "Quad" stain (Conn and Darrow[6]). Gross and longitudinal sections were made of both stretched and collapsed tracheas.

Three fresh and seven fixed tracheas were dissected with the aid of lowpower dissecting microscope and studied grossly.

The syringes from 9 turkeys were sectioned. Five were fixed in 10 per cent formalin and the other four in Zenker's fluid. The following stains were used: hematoxylin and eosin, Masson's, Heidenhain's "azan," Giemsa's, and Masson's trichrome. In 1 case, an entire lung was sectioned serially at 7 μ. In this serial sectioning, every twentieth section was placed on a slide and stained

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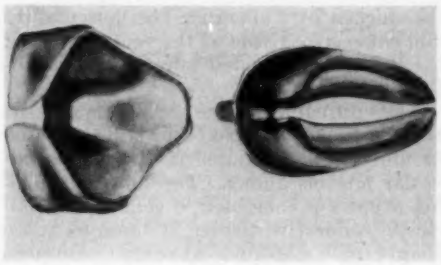

Fig. 1—Cartilages of larynx (drawings). Right—arytenoid cartilage dorsal view; left—cricoid cartilage dorsal view.

by the hematoxylin and eosin method. In addition, both fresh and embalmed lungs were dissected to study their gross structure.

Casts of the lungs and air sacs were made by intratracheal injections into recently killed birds as well as embalmed specimens. The embalming fluid was injected into the ischiatic artery by gravity.

A pressure of not over 100 mm. of mercury was used in making intratracheal injections. One thousand milliliters of injection fluid filled the system of the first adult turkey injected except for the small amount of air that remained in some of the air sacs. During subsequent injections, the birds were placed in different positions in order to distribute the air. The injection materials used were latex and vinyl resin.

EXPERIMENTAL RESULTS

The Larynx.—The larynx projects upward slightly into the cavity of the pharynx in the median plane. The aditus laryngis is slitlike. The epiglottis is represented by a short transverse fold of mucous membrane anterior to the aditus laryngis.

Two cartilages, the cricoid and arytenoid, form the framework of the larynx (fig. 1). The arytenoid cartilage has the appearance of a two-tined fork. The short thin handle forms an articulation on either side for the dorsal ends of the cricoid cartilage. The two prongs extend forward and downward. They are more massive than the handle and are convex dorsally. The anterior extremity is free but does not appear thus in the whole organ, since it is covered by muscle. The medial edges of these two prongs form the edges of the aditus laryngis; they are curled upward and outward. The space between the lateral edge of the prong and the cricoid cartilage is filled with muscular tissue. The prongs are thickened where they join the handle. The dorsal surface of this area is excavated for muscular attachment.

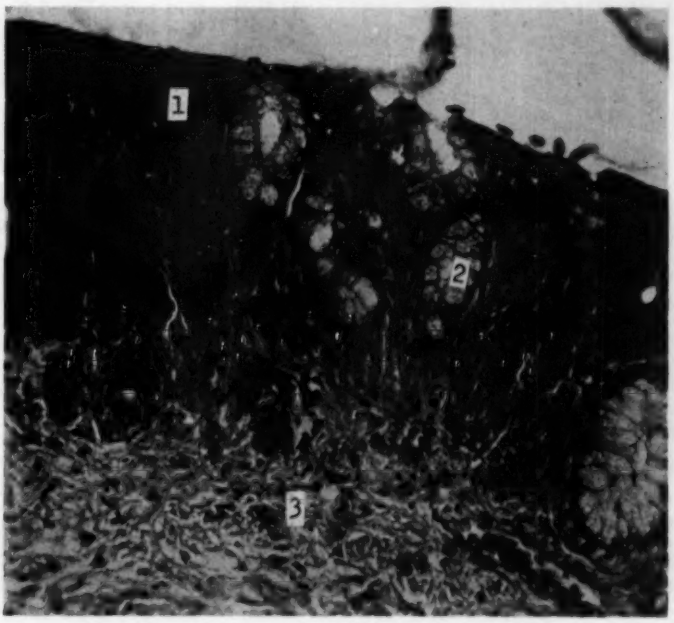

Fig. 2—Epithelium of larynx at area where stratified squamous changes to the glandular type; hematoxylin and eosin stain. x 300. 1—Stratified squamous epithelium; 2—mucous glands; 3—lamina propria.

The cricoid cartilage is flattened and ringlike but is not complete dorsally. At this point, the posterior projection or handle of the arytenoid cartilage completes the circle. The dorsal and lateral parts of the cricoid cartilage are flattened. Ventrally, however, there is an anterior projection which ends bluntly. Its greatest width is 1.3 cm. The ventral enlargement of the cricoid cartilage is ossified and possesses a tubercle on its medial surface. The short handle of the arytenoid cartilage is ossified in the adult birds.

Since the larynx projects slightly into the cavity of the pharynx, a part of its external surface is covered by a stratified squamous epithelium (fig. 2). This epithelium and the connective tissue which is found beneath it have been described in the chicken by Calhoun.[4] The musculature and connective tissue of the ventral part of the larynx are blended into that of the adjacent cervical area.

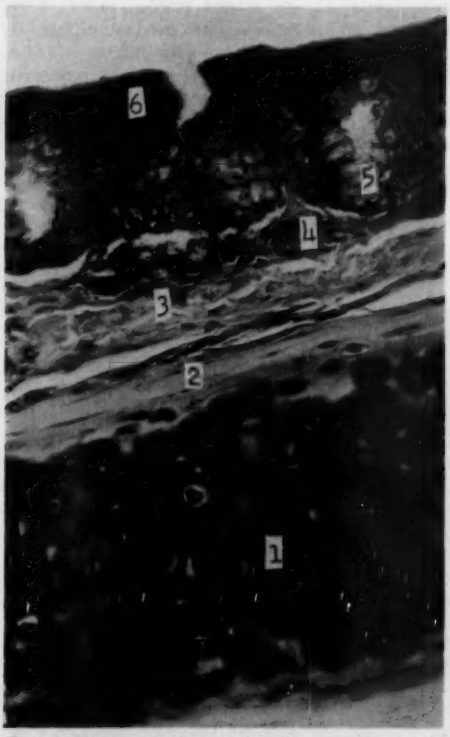

Fig. 3—Cross section of trachea; hematoxylin and eosin stain. x 300. 1—Hyaline cartilage of ring; 2—perichondrium; 3—lamina propria; 4—blood vessels in lamina propria; 5—simple alveolar mucous glands; 6—pseudostratified ciliated epithelium.

The epithelium of the trachea (fig. 3) is ciliated pseudostratified and is interrupted at almost regular intervals by simple alveolar mucous glands. The subjacent lamina propria is composed of collagenous and elastic connective tissue. It contains many simple or compound alveolar mucous glands and is very vascular. A more complete description of this mucous membrane will be found with the description of the trachea.

The supportive layer is less dense and less vascular than the lamina propria. The hyaline cartilages of the larynx are imbedded in this layer. The outer layer of the perichondrium blends into the connective tissue of the supporting layer. The cornua II of the hyoid bone is attached by means of connective tissue to the ventral surfaces of the larynx and the first few tracheal cartilages.

Vocal cords are absent. The internal surface is smooth except for the fold of mucous membrane produced by the dorsal tubercle of the cricoid cartilage and for a dorsal longitudinal median fold. The latter fold is produced by the handle-like projection of the arytenoid cartilage.

The first ring of the trachea is attached to the posterior edge of the cricoid cartilage by collagenous connective tissue.

The Trachea.—The course of the trachea does not follow that of the cervical vertebral column. At its anterior end it is median in position but, after approximately 3 in., it inclines to the right and lies ventrolaterally to the cervical vertebrae. It remains on the right side until it nears the thoracic inlet where it again becomes median in position.

The trachea is circular in cross section, and its lumen is maintained by the presence of 120 complete cartilaginous rings which overlap (fig. 4). The mucous membrane, the framework or supportive layer, and the external or adventitial layer constitute the three layers of the trachea.

The mucous membrane of the trachea is composed of an epithelium and a lamina propria (fig. 3). The epithelium is pseudostratified and ciliated, and it is interrupted at intervals by simple alveolar mucous

glands. There are only two types of epithelial cells in this pseudostratification. The deeper cells are pyramidal in shape and short, while the more superficial cells are tall and thin. The tall cells are enlarged superficially giving a fanlike appearance to the groups of cells between the mucous glands. The mucous glands are of the simple alveolar type. Occasionally, one gland is elongated sufficiently to become simple tubular. The alveolar glands have no distinct ducts, the glandular epithelium being replaced at the neck by the columnar cells of the pseudostratified area.

The lamina propria (fig. 3) is composed of dense, irregular connective tissue. It is richly supplied with blood vessels, most of which are capillaries. The bases of most of the simple alveolar mucous glands extend into the lamina propria but do not reach the supportive layer. The deeper parts of the lamina propria consist principally of collagenous connective tissue, which is directly continuous with the perichondrium of the tracheal rings.

The framework of the trachea is formed by 120 complete hyaline cartilaginous rings (fig. 3, 4). The cartilage, which has an outer and inner perichondrium, is thinnest at its edges. The inner surface of the ring is flattened while the outer surface is divided into two areas of equal size, one of which is convex and the other flattened. The change from a convex to a flattened surface occurs dorsally and ventrally (fig. 4). The convex and flattened areas alternate so that the convex surface of one overlaps the flattened area of the adjacent ring. This allows the trachea to change in length. The movement between rings is limited by the collagenous connective tissue.

External to the cartilage, there is a circular layer of collagenous connective tissue which attaches the trachea to the adjacent structures. On either side there is a thin, narrow striated muscle (trachealis) which parallels the trachea throughout its course (fig. 4). The muscle is partially attached to the outer fibrous layer of the trachea by rather loose and irregular collagenous fibers.

Fig. 4—Sketch of tracheal rings showing their relative position. Dorsal view. 1—Cartilaginous ring; 2—adventitia; 3—mucous membrane; 4—trachealis muscle.

Each of the last 12 tracheal rings becomes rounded ventrally (fig. 5) producing more intercartilaginous space. The rings remain flattened on the dorsal surface but do not overlap.

Syrinx and Extrapulmonary Bronchi.—The syrinx is a specialized structure which includes the last two tracheal and first eight bronchial cartilages. The last two tracheal cartilages are greater in circumference than any of the others but thinner

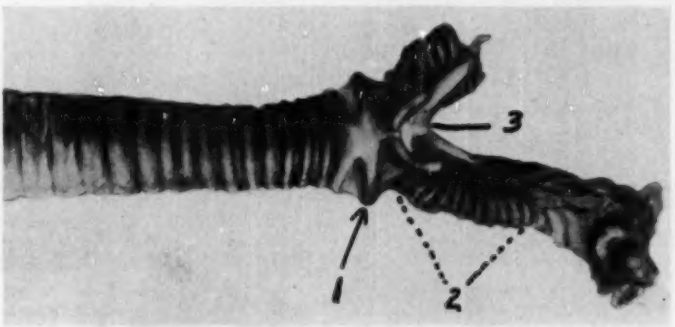

Fig. 5—Ventral view of syrinx and adjacent parts of trachea and bronchi (drawing). 1—First tracheal ring; 2—incomplete bronchial rings; 3—v-shaped mass of connective tissue.

in substance and are joined to each other ventrally (fig. 5). The ventral portion is ossified. The ventral part of the posterior edge of the last ring has a small projection which articulates with the first two bronchial cartilages by means of two facets. From the dorsal surface of the posterior projection, a vertical rod-shaped spike of bone projects upward to fuse with the posterior border of the last tracheal ring. This divides the trachea into halves and portends the origin of the two bronchi. On the posterior border of the last tracheal ring lateral to the spike (on each side), there is a facet for articulation with the first bronchial cartilage.

Each bronchus has its origin from the dorsal, lateral, and ventral parts of the last tracheal ring and from the rod of cartilage which divides the tracheal lumen. The bronchi, like the trachea, have a cartilaginous framework. They diverge from their origin, inclining laterally toward the lungs. A V-shaped mass of connective tissue is situated in the angle of divergence of the two bronchi (fig. 5). Its lateral edges are united to the medial surfaces of the bronchi where the bronchial cartilages are incomplete. The cartilages are round on cross section. The space between adjacent cartilages is equal to the width of each cartilage and is closed by a thin layer of connective tissue. The dorsal and ventral surfaces of the first two cartilages are enlarged and united with each other. At each of these two points, an irregular tubercle-like process projects forward to articulate with the facets of the last tracheal ring.

The other 11 bronchial rings are also incomplete medially, and each in succession becomes smaller and more deficient in substance ventrally. Thus, as the bronchus reaches the lung the cartilaginous framework disappears medially and ventrally.

The mucous membrane of the syrinx and extrapulmonary bronchi, like that of the trachea, consists of an epithelium and a lamina propria (fig. 6). In the most anterior part, the epithelium is pseudostratified ciliated. It becomes a two-layered, squamous, or low columnar epithelium over the large intercartilaginous spaces between the last two tracheal cartilages and also between the last tracheal and first bronchial

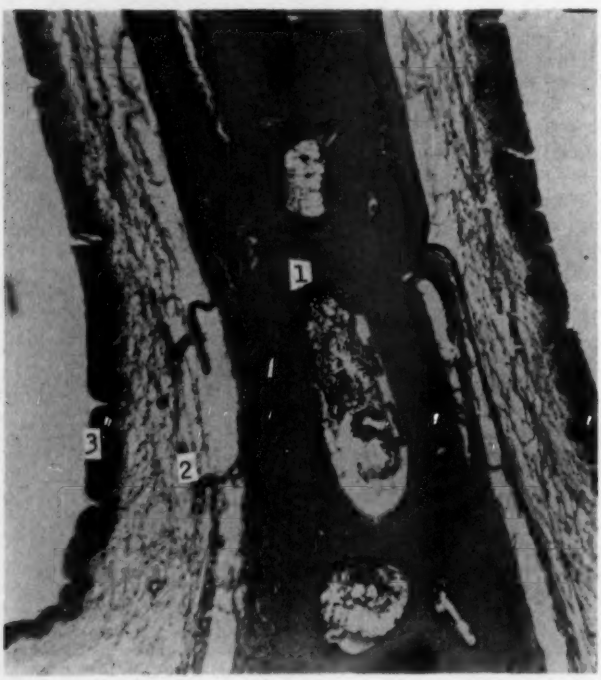

Fig. 6—Wall of syrinx showing ossified bar of cartilage which forms medial origin of bronchi, hematoxylin and eosin stain. x 75. 1—Ossified bar of cartilage; 2—lamina propria; 3—epithelium.

cartilage. The mucous glands are absent in the latter area. In the syrinx there are simple alveolar mucous glands, in the cells of which spherical vacuoles are found. The cavity of the gland is round and opens directly upon the surface. From this point to the vestibule, the epithelium gradually becomes taller until it is a ciliated simple columnar type. The lamina propria of the syrinx is thin and contains lymph nodules.

The adventitial layer is a thin layer of connective tissue which holds the syrinx in position. A portion of the air sac wall contacts the external surface of the middle third of the syrinx. Thus, the simple low cuboidal epithelium of the air sac becomes an external covering on this portion of the syrinx.

The framework of the syrinx and bronchi contains hyaline cartilage which has a well-marked perichondrium. Where the rings are incomplete and between the rings, only two layers, the mucous membrane and adventitia, are present.

Toward the ventrolateral aspect of the V-shaped collagenous connective tissue mass there are numerous irregular bands of smooth muscle fibers which connect the medial free ends of the bronchial cartilages (fig. 5).

The gross structure of the lungs follows in general the description given for the chicken by McLeod and Wagers.[15] For this reason it will not be described in detail. The lungs are held against the upper thoracic wall by connective tissue. The unit of lung structure is the lung lobule. These cylindrical six-sided lobules are similar to liver lobules in that they have a central longitudinal canal. The central lumen is that of the parabronchus or tertiary bronchus. The main or primary bronchus enters the ventral surface of the lung at the junction of its anterior and middle thirds. Just after its entrance the primary bronchus widens, forming a short vestibule. The intrapulmonary part of the primary bronchus gives off secondary bronchi which, in turn, give rise to the tertiary bronchi or parabronchi. The primary, secondary, and tertiary bronchi are conducting tubules. The respiratory tubules arise at right angles from each parabronchus. This mass of tubules is enclosed in the interlobular connective tissue. The parabronchi of the lung anastomose at variable intervals. Latex and vinylite corrosion specimens clearly show the anastomosing parabronchi which together produce the architecture of the lung (fig. 7).

Although hyaline cartilage is prominent in the extrapulmonary bronchus, it is gradually lost in the vestibule. Beyond the vestibule, the intrapulmonary bronchus is composed of the mucous membrane and the adventitia. The mucous membrane is like that of the trachea except for the distribution of the alveolar glands. The epithelium is pseudostratified ciliated. As the primary bronchus penetrates into the lung substance the glands decrease in size and number, and goblet cells appear in the epithelial layer. Lymph nodules which cause the

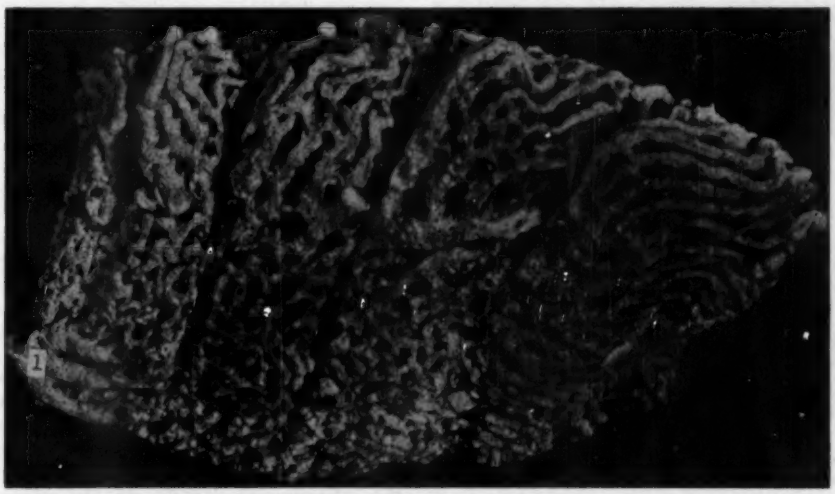

Fig. 7—Vinyl resin cast of left lung showing the anastomoses of the parabronchi on its lateral surface and the origin of the greater abdominal air sac (1). Anterior extremity of lung is to the right.

epithelium to bulge into the bronchial lumen are found in the loosely arranged and very vascular lamina propria. The adventitia consists of collagenous and elastic fibers and longitudinally arranged smooth muscle fibers.

The walls of the secondary bronchi consist of a mucous membrane and an adventitia. The epithelium is a ciliated simple columnar type. In parts of the secondary bronchus, there are a few simple alveolar mucous glands in addition to the goblet cells. In some areas, the epithelium is thrown into low circular folds by the subjacent lamina propria. The lamina propria is similar to that of the primary bronchus. The adventitial layer contains a few more smooth muscle fibers than that of the primary bronchus.

The walls of the tertiary bronchi or parabronchi are similar to those of the other conducting tubules (fig. 8). The mucous membrane, however, is formed of an inner simple squamous epithelium and an outer thin lamina propria (fig. 8). The inner part of the adventitia is composed of a layer of four to seven smooth muscle fibers arranged in a circular manner. Beyond this the adventitia consists of delicate, thin collagenous connective tissue.

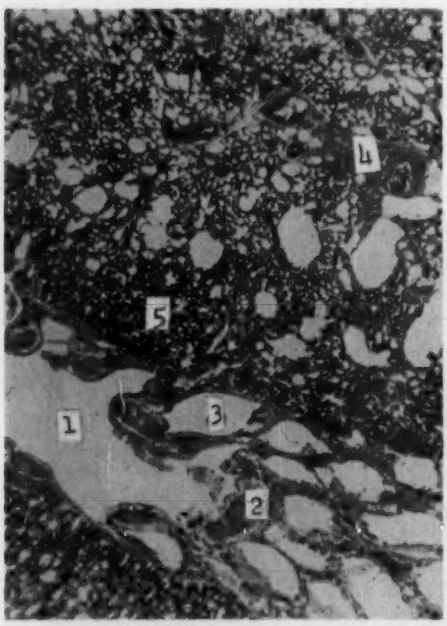

Fig. 8—Parabronchus oblique section, hematoxylin and eosin stain. x 75. 1—Lumen of parabronchus; 2—smooth muscle of parabronchial wall; 3—aperture into respiratory tubules cross section; 4—interlobular vessels and connective tissue; 5—area of respiratory tubules.

The respiratory tubules continue to branch into smaller and smaller structures (fig. 8), which end as narrow finger-like projections. The microscopic structure of the respiratory tubules is similar to that of the alveoli of the mammalian lung. Near the parabronchial aperture the epithelium of the tubules is simple cuboidal, then gradually reduces in height to become indistinct. Many erythrocytes are found within the walls of these small tubules, indicating the presence of many capillaries. Many respiratory tubules branch in all directions from each parabronchial aperture but do not anastomose with the adjacent ones. The tubules are of various lengths and begin at different distances from the aperture. The periphery of one lobule is separated from the adjacent lobules by a delicate connective tissue which contains both collagenous and elastic fibers.

DISCUSSION

The gross anatomy of the chicken's larynx has been described in several reports, and the larynx of the turkey is similar in construction. The author prefers the terms *larynx* for the anterior organ and *syrinx* for the posterior structure at the tracheal bifurcation. The turkey's larynx does not include vocal cords or an epiglottic cartilage. Cricoid and arytenoid cartilages are present. The arytenoid cartilage is a composite structure made up of what corresponds to the two (paired) arytenoid cartilages in mammals. This agrees in general with the description of the chicken's larynx by McLeod and Wagers.[15] They recognized the same two cartilages but indicated that a part of the thyroid cartilage had fused to the ventral portion of the cricoid cartilage. However, they did not mention the small handle-like process of the fused arytenoid cartilage which articulates with the incomplete dorsal portion of the cricoid cartilage.

A stratified squamous epithelium covers the pharyngeal surface of the pharynx. Just inside the aditus laryngis it becomes pseudostratified and ciliated.

No attempt was made to isolate the muscles of the larynx. It was suggested by Chauveau [5] that, in the chicken, the large muscle which encircles the aditus laryngis can completely close the opening, minimizing the lack of an epiglottis. It has also been suggested that the muscle of the larynx can definitely control the quantity of air which enters or leaves the trachea. The large sphincter muscle surrounding the aditus in the turkey could conceivably close the opening completely.

The trachea contains 120 hyaline cartilaginous rings. Their unique form and arrangement allow the trachea to elongate and shorten as well as to bend in almost any manner without any serious loss of function. All reports agree that the rings are complete in the chicken. Bradley states that the rings in the chicken are alternately large and small, and that the larger rings half overlap the smaller ones. In the turkey the tracheal rings are so arranged that, while each overlaps the two adjacent rings on one half of its circumference, it is overlapped by them on the other. The trachealis muscle,[15] which lies longitudinally along the trachea, is thus able to lengthen or shorten the trachea. No evidence of ossification was found in any of the cartilages of the trachea.

Batt [1] described in the chicken's trachea two types of epithelium, *viz.*, the columnar surface epithelium and the mucous cells in the glandular crypts. The author agrees with Batt that the mucous glands are a part of the epithelial layer.

The syrinx is considered to include the last two tracheal and the first eight bronchial cartilages. Microscopically, its structure parallels that of the trachea. The cells of the simple alveolar mucous glands, however, contain spherical vacuoles. Since the irregular bands of smooth muscle in the V-shaped mass of connective tissue are situated close to the open ends of the bronchial cartilages, it may be postulated that they can have an effect on the size of the lumen of the syrinx.

Several significant features concerning the structure of the lungs should be mentioned. The lungs are connected to the lateral thoracic walls by collagenous connective tissue. This feature was mentioned by Chauveau [5] and again by Batt,[1] but it appears that more recently investigators have overlooked this important point. Respiration by alternate expansion and relaxation of the lung substance is limited, since the organs are held tightly in place. The turkey's lung is relatively inelastic, since elastic fibers were found only in the wall of the conducting tubules. The use of the lung lobule as a unit of structure in describing the lungs seems to follow the general trend in histology. Batt also used the term *lung lobule* in his description. The disposition of the terminal respiratory tubules, however, is controversial. Sappey [16] stated that the canaliculi (respiratory tubules) anastomose. McLeod and Wagers [13] reported that the air cells are not blind but anastomose freely. Sisson [17] described the minute tubules of the avian lung as having numerous dilatations which correspond to the mammalian lung. This would indicate that the small tubules do not anastomose. Batt [1] also gave this same impression. Hazelhoff [12] gave a diagram of a parabronchus which showed the respiratory tubules to be a mass of blind finger-like projections from the parabronchus outward toward the periphery of the tubule. This is the type of structure that has been found in the turkey lung. No evidence of respiratory tubule anastomosis has been found, either in latex- or vinylite-injected specimens or in lung sections. However, anastomoses between parabronchi do occur and can be seen readily under low magnifications. The microscopic structure of the respiratory tubules is similar to that of the alveoli of the mammalian lung. However, as in the mammalian lung, because of the minuteness of the structures, the exact cytology of the thin respiratory walls is not clear.

Summary and Conclusions

The cricoid and arytenoid cartilages comprise the framework of the larynx. Its mucous membrane is composed of a ciliated pseudostratified epithelium which is interrupted at intervals by simple or compound alveolar mucous glands.

The wall of the trachea contains 120 complete cartilaginous rings. Each ring is overlapped by the adjacent rings on one side, and overlaps the adjacent rings on its other side. Three layers of the wall are discernible microscopically: the mucous membrane, the supportive layer, and the adventitia. The mucous membrane is

formed by two layers, the epithelium and the lamina propria. The epithelium is pseudostratified and ciliated. The simple alveolar mucous glands are almost entirely intraepithelial and interrupt the lining at regular intervals. The lamina propria consists of collagenous and elastic fibers and is very vascular.

The syrinx is formed at the tracheal bifurcation by the last two tracheal and the first eight bronchial rings. Its wall consists of three layers: the mucous membrane, the supportive layer, and the adventitia. A pseudostratified ciliated epithelium and a lamina propria comprise the mucous membrane, except that a thin, double-layered squamous epithelium is found at the level of the last tracheal and first bronchial rings. The glands of the syrinx and extrapulmonary bronchi are like those of the trachea except that their cells contain spherical vacuoles. The middle third of the syrinx is adjacent to the aggregate air sac. A low, simple cuboidal epithelium covers the organ at this point. A V-shaped mass of connective tissue and smooth muscle lies between the bronchi at their divergence. The last 11 extrapulmonary bronchial rings are deficient in cartilage ventrally as well as medially. These, plus the eight of the syrinx, constitute the total number of bronchial rings. The wall of the extrapulmonary bronchi is similar in structure to that of the syrinx.

The unit of lung structure is the lobule, which is formed by a parabronchus and its respiratory tubules. Each primary bronchus gives off secondary bronchi which, in turn, give rise to the tertiary bronchi or parabronchi and these to the final small respiratory tubules of the lung.

The wall of the primary bronchus has two layers, the mucous membrane and the adventitia. The mucous membrane consists of a ciliated pseudostratified epithelium upon which simple alveolar mucous glands empty, and a lamina propria which contains lymph nodules. The adventitia is formed of delicate connective tissue and many bundles of smooth muscle fibers.

The secondary bronchi are similar in structure to the primary bronchi except that the epithelium is gradually reduced to a ciliated simple columnar type. There are a few simple alveolar mucous glands in addition to goblet cells in the epithelial layer. The parabronchi are lined by a simple squamous epithelium.

The respiratory tubules branch repeatedly and end as narrow finger-like projections. They can be termed cylindrical alveoli. At the parabronchial aperture the epithelium of the tubules becomes slightly taller, then gradually reduces in height to become indistinguishable.

References

[1] Batt, H. E.: A Further Study of the Normal Histology of the Domestic Fowl. Rep., Ont. Vet. College, (1925): 24-30.

[2] Bechtel, H. E., and McLeod, W. M.: Latex in the Preparation of Corrosions of Mammary Glands. Am. J. Vet. Res., *6*, (1945): 17-20.

[3] Bradley, O. C.: The Structure of the Fowl. 3rd ed. Oliver and Boyd, London, (1950): 49-53.

[4] Calhoun, M. L.: The Microscopic Anatomy of the Digestive Tract of Gallus Domesticus. J. Sci., Iowa State College, *7*, (1933): 261-382.

[5] Chauveau, A.: The Comparative Anatomy of the Domesticated Animals, D. Appleton and Co., New York, N. Y., (1873): 475-483.

[6] Conn, H. J., and Darrow, M. A.: Staining Procedures. Part I, Sec. B, 3rd ed. Biotech. Publications, Geneva, N. Y., (1949): 18-19.

[7] Dotterweich, H.: Versuch über den weg der Atemluft in der Vogellunge. Zeischr. f. Vergl. Physiol., *11*, (1930): 271-284.

[8] Dotterweich, H.: Ein Weiterer Beitrag zur Atmungs Physiologie der Vögel. Zeischr. f. Vergl. Physiol., *18*, (1933): 803-809.

[9] Dotterweich, H.: Die Atmung der Vogel. Zeischr. f. Vergl. Physiol., *23*, (1936): 744-770.

[10] Ellenberger, W., and Baum, H.: Handbuch der Vergleichenden Anatomie der Haustiere. Springer-Verlag, Berlin, (1943): 1095-1102.

[11] Foust, H. L.: Diseases of Poultry. 2nd ed. Iowa State College Press, Ames, Iowa, 1948.

[12] Hazelhoff, E. H.: Structure and Function of the Lung of Birds. Poultry Sci., *30*, (1951): 3-10.

[13] Juillet, M. A.: Reserches Anatomiques, Embryologiques, Histologiques et Comparatives sur le Poumon des Oiseaux. Archiv de Zoöl., *5s9*, (1912): 207-371.

[14] Kaupp, B. F.: The Anatomy of the Domestic Fowl. W. B. Saunders Co., Philadelphia, Pa., 1918.

[15] McLeod, W. M., and Wagers, R. P.: The Respiratory System of the Chicken. J.A.V.M.A., *95*, (1939): 59-70.

[16] Sappey, J. M.: Recherches sur l'appareil Respiratorie des Oiseaux. Germer-Bailliere et Theses Sciences, Paris, (1847): 1-92. Fide., Chauveau, 1873.

[17] Sisson, S.: The Anatomy of Domestic Animals. 3rd ed. W. B. Saunders, Philadelphia, Pa., (1948): 942-943.

[18] Vos, R. J.: Uber den Weg der Atemluft in der Entenlunge. Zeitsch. f. Vergl. Physiol., *21*, (1934): 552-578.

Gross and Microscopic Anatomy of the Respiratory System of the Turkey
III. The Air Sacs

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IN GENERAL all reports agree on the number of the chicken's air sacs (4 paired and 1 single), but they have often been named differently.

McLeod and Wagers [19] summarized the various names used and proposed a new series which seems suitable. The names suggested were: for the single anterior sac, anterior thoracic; for the paired sacs, thoraco-cervical, posterior thoracic, greater abdominal, and lesser abdominal. Bradley [5] reported that the clavicular sac (anterior thoracic) communicated with the lung through two openings. He also said that this sac pneumatizes the sternum, sternal ribs, shoulder girdle, and humerus. Foust,[14] Kaupp,[18] and Sappey [20] agreed with this. McLeod and Wagers [19] stated that this sac communicated with each primary bronchus as it pierced the diaphragm, and also communicated with the ventrobronchial system by recurrent bronchi. The sternal and humeral prolongations did not pneumatize the sternal ribs and coracoid bones.

Sappey [20] described the thoraco-cervical air sacs of the chicken in great detail. He was the only investigator to mention the intraspinal connections of the cervical and dorsal vertebrae and vertebral ribs. Kaupp's [18] description of this sac was very similar to that of Sappey. Bradley [5] agreed with these two authors in regard to the bones pneumatized by it. McLeod and Wagers [19] were the only investigators to show the pulmonary origin of this sac. They found that it communicated with the anterior ventral bronchus, ventral to the first rib. The descriptions of the posterior thoracic sac are in general agreement. However, Kaupp and Sappey [18, 20] both stated that this sac had a double communication with the lung, while McLeod and Wagers [19] mentioned only one. Regarding the lesser abdominal sacs, Kaupp and Sappey [18, 20] agreed with McLeod and Wagers [19] on almost all points, including their origin from the primary bronchus at the posterior extremity of the lung. There was, however, some controversy regarding the connections of the air sacs with the femur. Kaupp [18] found extensions into the femur in the chicken and Sappey [20] observed them in some other birds. McLeod and Wagers [19] reported pneumatization of the sacrum, coccygeal vertebrae, and the ilia of the chicken but not the femur.

Brandes [4] reported on the extent and location of the air sacs in birds.

Sappey [20] discussed the microscopic structure of the air sacs. He stated that the walls were essentially formed by a thin celluloserous membrane strengthened in some places by an external envelope of elastic fibrous tissue. Long, thin blood vessels were distributed to the substance of these walls. McLeod and Wagers [19] stated that the sacs were mucoserous structures.

Bradley [5] stated that the air sacs appeared in the chick during the first week after hatching. He also noted that the cavities of the developing bones which later become pneumatic were first filled with marrow.

MATERIALS AND METHODS

Casts of the lungs and air sacs were made by intratracheal injection of latex or vinyl resin into recently killed birds as well as of embalmed specimens. The embalming fluid was injected into the ischiatic artery by gravity.

A pressure of not over 100 mm. of mercury was used in making intratracheal injections.

One thousand milliliters of injection fluid filled the system of the first adult turkey injected, except for a small amount of air that remained in some of the air sacs. During subsequent injections, the birds were placed in different positions in order to distribute this air.

An additional means of avoiding the resistance of the residual air to injection was the drilling of a small hole into the keel of the sternum and another into the distal extremity of the humerus. This allowed the contained air to be forced out. As the injection progressed, the fluid eventually found its way to these artificial openings. They were then plugged with wooden or metal pins and the injection continued to its termination. In this fashion, relatively complete injection into the pneumatized humerus, sternum, and air sacs, which are connected with these pneumatic bones, was accomplished.

After the injection was completed, the trachea was ligated and the cadavers were set aside for forty-eight to ninety-six hours to permit thorough hardening of the injection mass. The vinyl resin material set more rapidly than the latex mass. In the case of latex injection into unembalmed birds, 100 ml. of a 5 per cent acetic acid solution was

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forced into the respiratory system just before the mass injection was done. This solution acidified the surfaces of the respiratory tracts adequately to assure the setting of the latex material.

The respiratory organs of several of the injected turkeys were dissected. The remaining casts were freed of tissue by maceration or by corrosion with strong acids.[2]

In all, 7 adult turkeys were injected with latex and 4 with vinyl resin. The posterior thoracic air sac ruptured in two of the latex-injected specimens.

The casts of vinyl resin became hard and warped after exposure to the air. The latex casts, on the other hand, remained soft and pliable except when they were exposed to concentrated hydrochloric acid.

Five, freshly killed adult turkeys were injected intratracheally with plaster of paris. The mixture was allowed to flow into the respiratory tract by gravity from a height of 3 ft. The turkeys were allowed to remain at room temperature for three hours and were then placed in a freezing chamber at −17 F. After they had frozen, 3 of the turkeys were cut in half along the midline. The other 2 were dissected as complete specimens. This technique produced well-formed casts of the larger tubular and saccular structures but failed to fill completely all of the small diverticula and extensions.

Following this experiment, 3 turkeys were treated in the following manner. The trachea was cannulated, and 1,000 ml. of Zenker's solution to which 20 Gm. of barium antimonyl tartrate had been added was injected by gravity. The trachea was ligated following this infusion and x-rays were taken. The x-rays served to confirm the gross anatomical findings.

Latex intratracheal injections were also made into 6 turkeys 1 day of age, 6 turkeys 3 days of age, 6 turkeys 5 weeks of age, 3 turkeys 11 weeks of age, and 3 turkeys 17 weeks of age. Similarly vinyl resin injections were made into 6 turkeys 1 day of age, 6 turkeys 3 days of age, 3 turkeys 5 weeks of age, 4 turkeys 11 weeks of age, and 2 turkeys 17 weeks of age.

The procedure differed slightly in the 1- and 3-day-old birds. The respiratory system was injected via the trachea by a rubber tube connected to a 10-ml. syringe. The poult cadavers were set aside for twelve to forty-eight hours in order to allow the injection mass to harden. They were then either dissected or macerated. The older poults were injected by the method used in the adult birds.

Transverse, 10 μ sections were made through the cervical, thoracic, and abdominal regions of 12 day-old poults by the celloidin technique,[3] and stained with hematoxylin and eosin.

Sections of the cervical vertebrae from 2 adult birds were processed in this same manner except

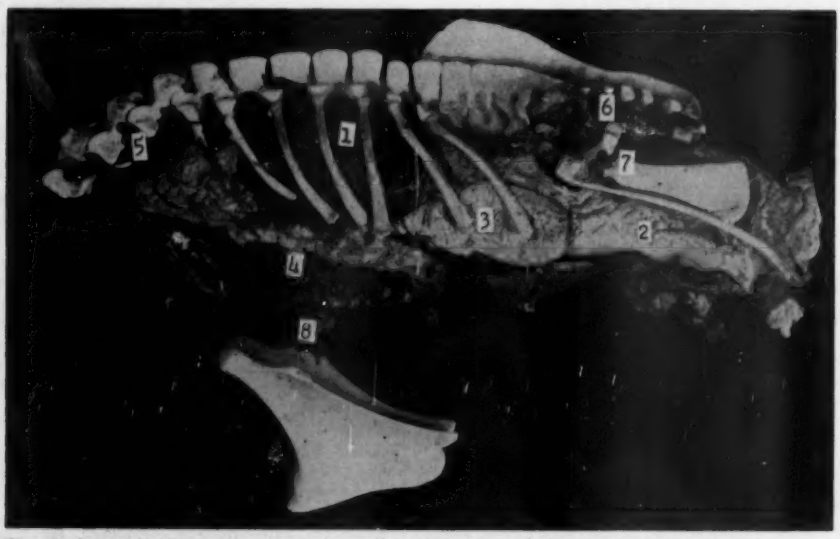

Fig. 1—Lateral view of macerated specimen of 11-week-old turkey which had been injected intratracheally with vinyl resin. The ilium of the left side has been removed to show suprarenal diverticula. 1—Lung; 2—greater abdominal air sac; 3—lesser abdominal air sac; 4—aggregate air sac; 5—cervical prolongation of aggregate air sac; 6—suprarenal diverticulum of posterior abdominal air sac; 7—femoral diverticulum of posterior abdominal air sac; 8—sternal prolongation of aggregate air sac.

that the bones were decalcified by the electrolytic process described by Ducey.[12]

Sections from various areas of the air sacs of 7 turkeys were prepared by the paraffin method. They were stained by several techniques, including hematoxylin and eosin, Heidenhain's "azan," and Gomori's aldehyde fuchsin stain for elastic tissue.[13]

EXPERIMENTAL RESULTS

The names of the nine air sacs are the anterior thoracic which is unpaired, and the posterior thoracic, thoraco-cervical, lesser abdominal, and greater abdominal, which are paired.

The paired posterior thoracic and thoraco-cervical as well as the single anterior thoracic air sacs are combined into a single large compartment, the aggregate sac (fig. 1), which communicates with the air passageways of the lung at its anteroventral border. There are two unions, one with a ventral bronchus and another with an area of several parabronchi. The combined sacs have the same location and visceral relations as the individual sacs which were described by McLeod and Wagers.[10] In addition, however, numerous diverticula are present. One of these is a large ventral diverticulum which pneumatizes the sternum through several foramina on its dorsal surface.

From the dorsal surface a diverticulum extends into each transverse canal of the cervical region as far forward as the second cervical vertebra (fig. 1 and 2). In the spaces between the transverse foramina, the cervical extension expands so that it covers much of the lateral surface of each vertebra. From each expanded portion, an anterior prolongation extends upward and forward between the anterior articular process and the spine (fig. 2), where it joins the one from the opposite side. A posterior prolongation from the expanded area extends under the posterior articular process to the intervertebral space where it joins the one from the opposite side. A small, pointed projection extends forward from each junction to the spine of the preceding vertebra. An anastomosing radicle passes from the junction through the vertebral canal dorsal to the spinal cord (fig. 2).

As the cervical extension passes through the transverse canal it sends off radicles which enter each vertebra through foramina in its body. A continuation of the posterior part of the cervical extension passes caudally along the sides of the vertebrae as far as the fourth coccygeal. It produces an anastomosing network about each vertebra similar to that in the cervical region. However, in those areas where the vertebrae are fused, the anastomosis between the two sides was not evident since it occurs within the fusion. Each thoracic and coccygeal vertebra has numerous pneumatic foramina around the transverse process, while each vertebral segment of the synsacrum is invaded by the air sac extension through an opening near the intervertebral foramen. Each vertebral rib is pneumatized through several foramina on the posterior surface of the body. The posterior vertebral continuation receives a

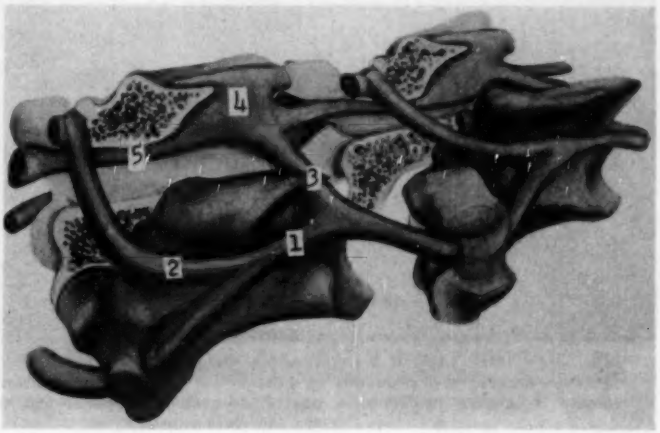

Fig. 2—Cervical vertebrae which are split on midline showing the position of the air sac prolongations about the vertebrae and through the vertebral canal. 1—Cervical extension; 2—anterior prolongation; 3—posterior prolongation; 4—dorsal confluence; 5—intraspinal connection.

connection from the aggregate sac in the area of the last cervical vertebra and in the area of the first thoracic vertebra. In the region of the synsacrum, there is suprarenal diverticulum which communicates with the posterior vertebral continuation at every vertebral segment.

There is an extension from the dorsal part of the aggregate sac which courses from the thoracic cavity with the brachial vessels, crosses the axillary space, and pneumatizes the humerus through a large foramen on its shaft just below the head. It also has a small subscapular diverticulum.

The greater abdominal air sac arises on the most lateral aspect of the posterior extremity of the lung (fig. 3) by the confluence of 20 to 30 tertiary bronchi. The suprarenal diverticulum extends into the synsacrum. This is shown by the presence of injected material in the interior of the bones which form this structure (fig. 4). The latex was found in the three pelvic bones. The ilium is pneumatized at various points. One area is at the articulation (fused in the adult bird) between the ilium and the vertebrae. The suprarenal diverticulum also enters the ilium through small foramina in the concave caudal area of its medial surface. The pubis is pneumatized from the ischium at the acetabulum. The ischium receives a prolongation of the air sac through several small foramina, in the triangular depression on the lateral surface just posterior to the acetabulum and in front of the ischiatic foramen. It is also pneumatized through several small foramina on the concave portion of its medial surface.

There are two coxofemoral diverticula. The anterior one is small. It reaches the anteroventral edge of the acetabulum by winding outward and upward over a notch on the acetabulum. The larger posterior diverticulum is found just caudal to the acetabulum. It reaches this position by passing through the ischiatic notch. These two diverticula embrace the joint but do not pneumatize the femur.

Each of the paired lesser abdominal air sacs is located between the body wall and the anterior portion of the greater abdominal air sac of the same side. Here the sternal end of the last rib crosses the sac about its middle. The position of the sac causes it to be compressed laterally. Communication with the lung occurred in two places. One connection is an extension of one of the dorsal secondary bronchi at the hilus of the lung. The other is dorsal to the first at the anterior portion of the sac and is formed by the confluence of numerous tertiary bronchi.

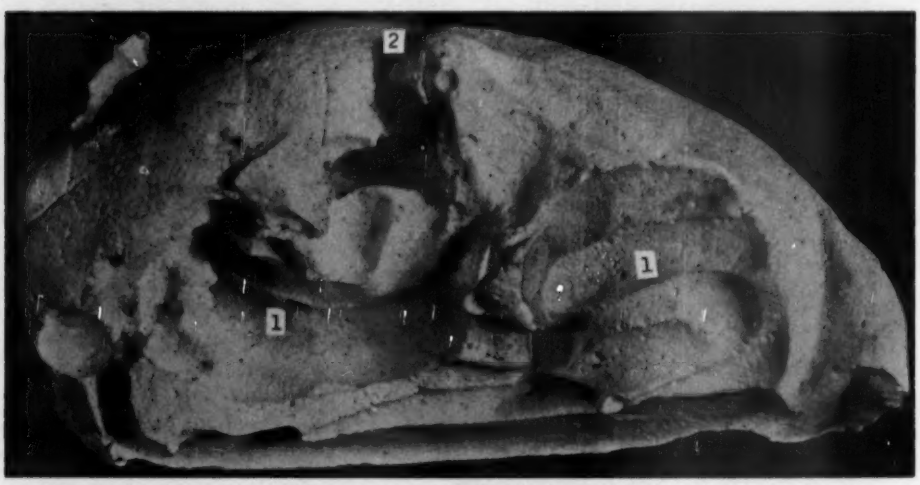

Fig. 3—Plaster cast of right greater abdominal air sac (medial view) showing the grooves and furrows produced by pressure of the adjacent viscera. 1—Grooves made by small intestines (mesenteric part); 2—groove produced by terminal part of small intestine.

Latex and vinylite were not found in any of the bones of the injected 1-day-old and 3-day-old poults. The bones of these poults are either entirely cartilaginous or just beginning to ossify. In day-old poults, the yolk sac occupies much of the space which is eventually assumed by the greater abdominal air sac but did clearly show the presence of all the air sacs. In 5- and 8-week-old turkeys, the injected materials were found in the sternum and thoracic vertebrae, while in those 11 and 17 weeks old the material was found also in the ribs, ossa coxarum, and the cervical, lumbar, and sacral vertebrae.

Microscopically, the wall of the air sacs consists of three layers, the inner epithelium, the connective tissue layer, and the outer epithelial covering. The air sacs adjacent to their pulmonary connection are lined by a simple ciliated columnar epithelium (fig. 5). Further away from this connection, the epithelial layer gradually becomes a simple squamous type. The connective tissue layer consists of irregular bundles of connective tissue, most of which are composed of elastic fibers. Blood vessels and an occasional solitary lymph nodule are found in this layer. The outer epithelial layer is simple squamous. No mucous glands of any type are found in the walls of the air sacs.

Discussion

Chauveau,[7] Kaupp,[18] and McLeod and Wagers [19] reported that the greater abdominal air sac of the chicken was a continuation of the primary bronchus at the posterior extremity of the lung. In the turkey, this sac arises from a number of parabronchi grouped at one point. McLeod and Wagers [19] stated that the lesser abdominal air sac originates from a ventral secondary bronchus. In the turkey, this sac has two connections with the lung, one to a secondary bronchus and the other by means of parabronchi. The three anterior air sacs are represented as diverticula of a single compartment. The presence of the vertebral diverticula and their prolongation into the spinal canal verifies the work done many years ago by Sappey.[20] It has apparently not been mentioned or verified since this initial report.

There has been some controversy concerning the pneumatization of the femur in the chicken. The injection material did not enter the femur in any of the turkeys used in these experiments.

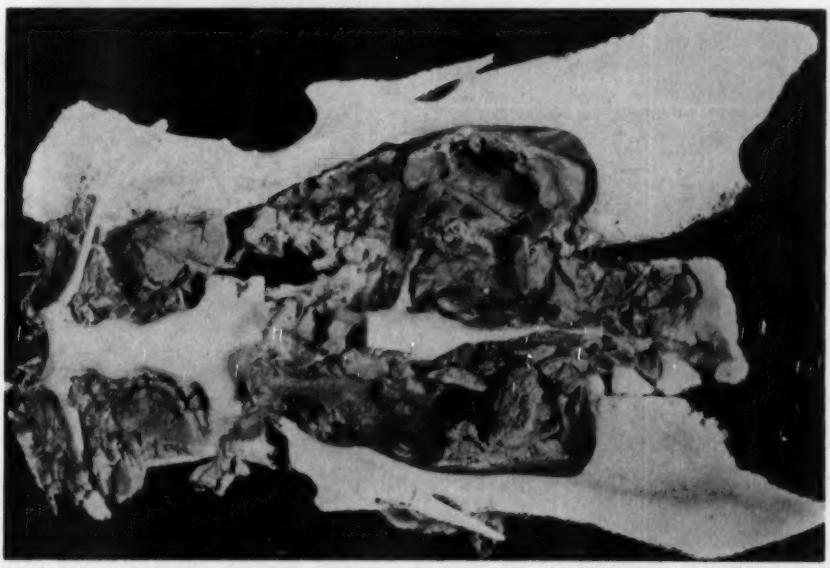

Fig. 4—Ventral view of synsacrum and ossa coxarum showing the suprarenal diverticula of greater abdominal sacs.

It is evident that the pneumatization of bones occurs gradually with maturation. The exact time that the air sac diverticula enter the bones has not been established. However, the sternum and thoracic vertebrae are pneumatized in 5-week-old turkeys, and at 11 weeks of age the ribs, ossa coxarum, and the cervical, lumbar, and sacral vertebrae were found to be pneumatized.

The method of injecting latex and vinylite used in this investigation was quite satisfactory. The operator could observe the rapidity of the injection and adjust the pressure accordingly. In only 2 cases did the air sacs burst under pressure. Acid corrosion of latex specimens is a more rapid method of cleaning the casts than maceration but does not produce as flexible nor as soft a cast. Clorox and other house-

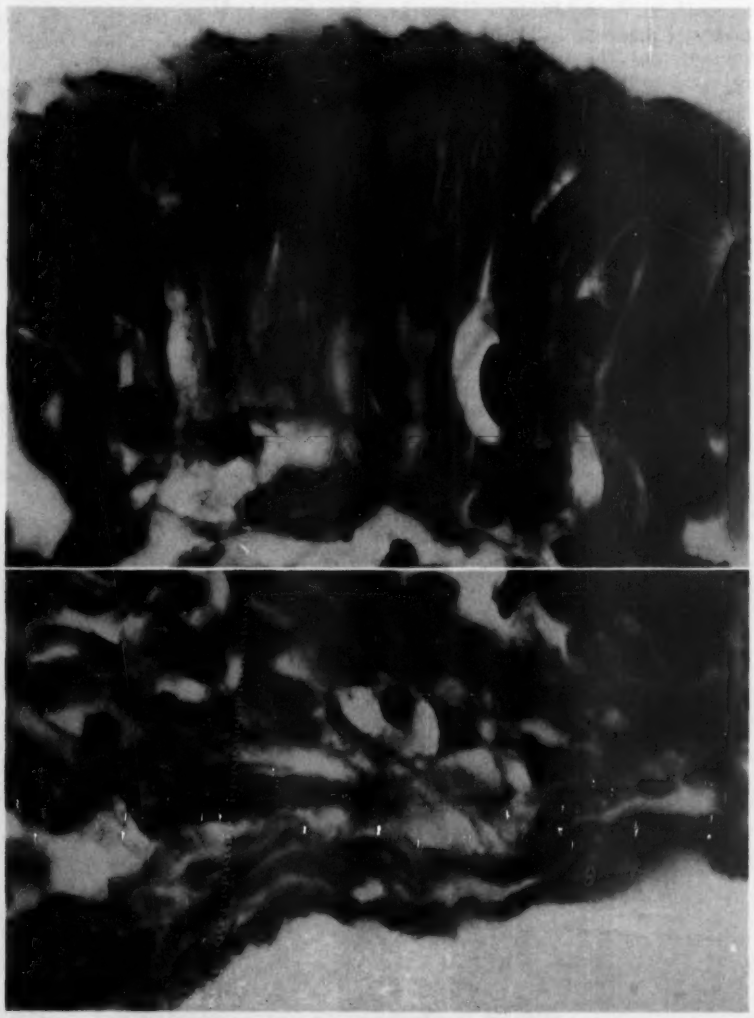

Fig. 5—Greater abdominal air sac showing a ciliated columnar epithelial lining (top) and the simple squamous outer epithelial layer. Between these two layers there is a connective tissue layer, hematoxylin and eosin strain. x 1000.

hold hypochlorite compounds were used to remove objectionable odors from macerated specimens.

Sappey [20] mentioned the possibility that some of the cranial bones are pneumatized. Although the intratracheal injections made in this investigation did not extend beyond the third cervical vertebra, the author believes that the bones which form the posterior cranial wall are pneumatized from the middle ear. A few latex injections were made through the external ear (after puncture of the tympanic membrane), and the material was found in the occipital and sphenoid bones. Investigation of the pneumatization of cranial bones will be made in the near future.

Summary and Conclusion

The nine air sacs are thin fibrocellular extensions from the lung parenchyma. The single anterior thoracic and the paired posterior thoracic and thoraco-cervical sacs connect with the air passageways of the anteroventral borders of the lung at two points, one with a ventral bronchus and another with an area of several parabronchi. These sacs are represented in the turkey as a single compartment. The paired greater abdominal air sacs arise on the most lateral aspect of the lung by the confluence of 20 to 30 tertiary bronchi. The paired lesser abdominal air sacs communicate with the lung in two places. One connection is with a dorsal secondary bronchus at the hilus of the lung, while the other is dorsal to the first and is formed by the confluence of numerous tertiary bronchi.

An air-sac diverticulum (vertebral) extends from the second cervical to the fourth coccygeal vertebra, lateral to the vertebral column. Three connections are made with this structure from the aggregate sac, and a series of communications arises from the suprarenal diverticulum of the greater abdominal air sac. The long vertebral diverticulum also sends prolongations over the vertebrae at the intervertebral areas and into the vertebral canal. There is also a coxofemoral diverticulum from the greater abdominal air sac.

The pneumatization of bones in 11-week-old poults is the same as in the adult. Only the humerus and sternum have air sac connections in 5-week-old poults.

The wall of the air sac is formed of an inner epithelium, a connective tissue layer, and an outer serous membrane. The inner epithelium is a ciliated simple columnar type near the pulmonary connections of the air sac but gradually changes to a simple squamous variety distally. The connective tissue layer, which contains blood vessels and occasional solitary lymph nodules, is composed primarily of elastic fibers. The outer serous membrane is formed by simple squamous epithelium. There are no mucous glands in the air sac wall.

References

[1] Batt, H. E.: A Further Study of the Normal Histology of the Domestic Fowl. Rep. Ont. Vet. College., (1925): 24-30.

[2] Bechtel, H. E., and McLeod, W. M.: Latex in the Preparation of Corrosions of Mammary Glands. Am. J. Vet. Res., *6*, (1945): 17-20.

[3] Bensley, R. R., and Bensley, S. R.: Handbook of Histological and Cytological Technique. Univ. of Chicago Press, Chicago, (1941): 140-141.

[4] Brandes, G.: Beobachtungen und Reflexionen über der Atmung der Vögel. Arch. f. d. ges. Physiol., *203*, (1924): 492-511.

[5] Bradley, O. C.: The Structure of the Fowl. 3rd ed. Oliver and Boyd, London, (1950): 49-53.

[6] Calhoun, M. L.: The Microscopic Anatomy of the Digestive Tract of Gallus Domesticus. J. Sci., Iowa State College, *7*, (1933): 261-382.

[7] Chaveau, A.: The Comparative Anatomy of the Domesticated Animals. D. Appleton & Co., N. Y., N. Y., (1873): 475-483.

[8] Conn, H. J., and Darrow, M. A.: Staining Procedures. Biotech. Publications, Geneva, N. Y. Part I, Sec. B, (1949): 18-19.

[9] Dotterweich, H.: Versuch über den weg der Atemluft in der Vogellunge. Zeischr. f. Vergl. Physiol., *11*, (1930): 271-284.

[10] Dotterweich, H.: Ein Weiterer Beitrag zur Atmungs Physiologie der Vögel. Zeischr. f. Vergl. Physiol., *18*, (1933): 803-809.

[11] Dotterweich, H.: Die Atmung der Vögel. Zeischr. f. Vergl. Physiol., *23*, (1936): 744-770.

[12] Ducey, E. F., and Shippey, R. T.: Decalcification of Bone by Electrolysis. Am. J. Clin. Path., *20*, (1950): 85-86.

[13] Ellenberger, W., and Baum, H.: Handbuch der Vergleichenden Anatomie der Haustiere. Springer-Verlag, Berlin, (1943): 1095-1102.

[14] Foust, H. L.: Diseases of Poultry. 2nd ed. Iowa State College Press, Ames, Iowa, 1948.

[15] Gomori, G.: Aldehyde-Fuchsin: A New Stain for Elastic Tissue. Am. J. Clin. Path., *20*, (1950): 665-666.

[16] Hazelhoff, E. H.: Structure and Function of the Lung of Birds. Poultry Sci., *30*, (1951): 3-10.

[17] Juillet, M. A.: Reserches Anatomiques, Embryologiques, Histologiques et Comparatives sur le Poumon des Oiseaux. Archiv de Zoöl., *5e9*, (1912): 207-371.

[18] Kaupp, B. F.: The Anatomy of the Domestic Fowl. W. B. Saunders Co., Philadelphia, Pa., 1918.

[19] McLeod, W. M., and Wagers, R. P.: The Respiratory System of the Chicken. J.A.V.M.A., *95*, (1939): 59-70.

[20] Sappey, J. M.: Recherches sur l'appareil Respiratories des Oiseaux, (1847): 1-92. Germer-Bailliere et Theses Sciences, Paris. Fide., Chauveau, 1873.

[21] Sisson, S.: The Anatomy of Domestic Animals. 3rd ed. W. B. Saunders, (1948): 942-943.

[22] Vos, H. J.: Uber den Weg der Atemluft in der Entenlunge. Zeitsch. f. Vergl. Physiol., *21*, (1934): 552-578.

A Study of Turkeys Artificially Infected with *Salmonella Typhimurium*

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DURING the past fifteen years, there have been numerous reports of Salmonella infections in poultry.

Several investigators [1, 2] consider poultry a potential reservoir of this infection for various mammals. Hinshaw [3, 4] has described the ubiquitous nature of Salmonella in animals and reported that man may serve as a carrier to infect poultry.

In studies of market birds, Felsenfeld, Young, and Yoshimura [5] found 9.2 per cent of the poultry from uninspected plants harbored some type of Salmonella. Schnieder and Gunderson [6] examined 1,014 eviscerated apparently normal chickens at random in one poultry plant and found 4.4 per cent of the birds to be positive for one or more species of Salmonalla organisms. During a recent investigation of Salmonella [7] in apparently healthy turkeys of market age, visceral samples from 27 turkeys (9 %) of 300 cultured were positive for one or more Salmonella.

A number of experiments have been conducted by feeding Salmonella organisms to turkeys. Lee *et al.*[8] isolated paratyphoid organisms from the infected ovules of turkey hens that had been infected with Salmonella by crop instillation. Gauger and Greaves [9] isolated *Salmonella typhimurium* from the gall bladder, ova, lungs, liver, kidney and intestinal tissues of inoculated poults. Later, Gauger and Greaves [10] orally inoculated adult birds with *S. typhimurium* and recovered the organism from the feces sixty-five days after inoculation. The organism was isolated from 1 of 6 birds in cultures taken from the tissues. The positive culture came from a diseased ovum. These same authors [11, 12] were able to demonstrate water contamination from adult birds infected by inoculation and infection of the tissues of poults fed Salmonella.

The objective of the experiment to be reported was to determine whether the carrier state developed among turkeys which were exposed to oral infection with *S. typhimurium*.

MATERIALS AND METHODS

The *S. typhimurium* strain used in the experiment was provided by Dr. P. R. Edwards, U. S. Public Health Service Laboratory, Chamblee, Ga. It was inoculated intraperitoneally into 1 poult. After the poult died, bits of liver were seeded into tryptose phosphate broth (Difco). After five hours of incubation at 37.5 C., streaking of the culture on 10 per cent serum agar yielded only smooth colonies of *S. typhimurium*. The liver was again seeded into tryptose phosphate broth and incubated for eighteen hours. The culture corresponded in turbidity to McFarland tube 4.

Fifty 6.5-week-old poults were inoculated with 1 cc. of the eighteen-hour broth culture by direct introduction into the crop with a 5.0-ml. pipette. After inoculations, the poults were kept in wire pens in an enclosed cubicle for three weeks. Then they were placed on deep litter in the same cubicle. Control poults of the same age were kept in another cubicle in the same building under similar conditions.

Before placing the poults in the room, the entire premises were washed with water, cleaned with steam at approximately 100-lb. pressure, and finally disinfected with a 3 per cent lye solution. A foot bath containing 5 per cent lye solution for cleaning rubber boots was placed at the door of the contaminated room.

The deep litter was composed of Canadian peat moss which was originally packaged in large paper bags. All feed was purchased in new feed sacks. The feed was kept in large metal rodent proof containers or in a rodent-proofed feed room.

Bacteriological examination of the tissues and feces was performed by several different methods. Enrichment mediums for tissue studies included tryptose phosphate, selenite F, and tetrathionate broths. Selective mediums used included MacConkey, bismuth sulfite, Levine E.M.B., S.S., and brilliant green agars. Only selenite and tetrathionate broths were used for enrichment for the fecal cultures. After several preliminary trials with the above mediums, all primary enrichments were made from tetrathionate and selenite broths. Subcultures from tetrathionate broth were made on brilliant green agar. S.S. agar was streaked from the selenite enrichment broth. The latter pairs of mediums and the enrichment seemed to offer greater facility of Salmonella isolation.

Tissues were taken at autopsy using aseptic techniques. Approximately 1 Gm. of tissue, minced with a sterile scissors, was placed in each enrichment broth.

For culturing feces, a piece of the cecum including the cecal tonsil and about ½ Gm. of feces were used to seed the enrichment mediums.

Cultures were made from bits of feathers, a toe, dust from cross slats above the birds' heads, and bits of litter gathered at random on the floor. All cultures were incubated for eighteen hours at 37.5

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C. in the enrichment broths and transfers made to the selective agars.

Typical colonies were picked and tested with *S. typhimurium* antiserum for susceptibility to agglutination. Some known positive cultures were used during each series of isolations in control agglutination tests against Salmonella group serums (A to E).

Results

Examinations of the poults were made twice daily for the five days following exposure. They did not manifest any visible symptoms of disease. At the fifth day, cultures were taken from the tissues and feces of 3 birds. *Salmonella typhimurium* was present in the feces but not in the tissues examined. The organism was present in spleen and feces of 5 birds at ten days after exposure. Other tissues were negative for *S. typhimurium*. Three nonexposed control birds were negative for Salmonella at this time. Nineteen days after oral inoculation, 5 birds were examined. *Salmonella typhimurium* was present in the liver, spleen, and feces of 2 birds. Culture of 3 more nonexposed control birds in another pen revealed *S. typhimurium* organisms in the lungs and spleen. At no time during this experiment did the control birds exhibit any symptoms of disease.

Cultures taken from the originally inoculated birds during the next two weeks were negative. Cultures were made of the feathers, dust, litter, and feet for several more weeks. *Salmonella typhimurium* was cultured from the feathers thirty-seven days after inoculation. The litter was positive for Salmonella forty-four days after feeding the organism. Viable organisms were present in the dust and on the birds' feet in contaminated pens seventy-one days after inoculating the poults. At forty-four days postinoculation, *S. typhimurium* was cultured from the feces of an artificially infected poult. *Salmonella typhimurium* were isolated from the liver, spleen, lung, and kidney of a control poult forty-four days after the original poults were infected. A fecal culture of the control poult at this time was negative. The dust in two control pens was positive for *S. typhimurium*.

The organism was shown to be capable of infecting poults by the intranasal route. Mortality resulted from intravenous and intraperitoneal inoculations.

Discussion

The results of this investigation indicate that turkeys may become infected with *S. typhimurium* without showing clinical symptoms. Tissue infection seems to be transitory. However, the intestine may yield the organism for a period of time. These findings are in agreement with the work of Pomeroy and Fenstermacher[13] and the studies of Gauger and Greaves.[12]

The organism was present on the feathers and in the dust up to seventy-one days. The dust seemed to be the most likely means of transmission of *S. typhimurium* from infected to control birds.

From this work and that of other workers, it would appear that the control of Salmonella in market birds might logically be directed toward a general sanitation program in the eviscerating plants and toward removal of organs of localization by Salmonella within the bird. This program might embrace complete evisceration of the birds and possibly a final thorough internal and external washing of the carcass with a chlorinated water as suggested by Goresline *et al.*[14]

Summary

1) *Salmonella typhimurium* was isolated from the dust, litter, feathers, feet, feces, kidney, liver, lung, and spleen after 71, 44, 37, 71, 44, 19, 19, 19, 19 days, respectively, following oral inoculation.

2) Uninoculated controls in another pen became infected, possibly through airborne organisms.

References

[1] Barnes, L. A.: Pathogenic Enteric Bacilli. U. S. Naval Bull., *43*, (1944): 939-949.

[2] Edwards, P. R.: Incidence of Salmonella Types in Fowls in the United States. Proc. World's Poult. Cong., (1939): 271-274.

[3] Hinshaw, W. R., and McNeil, E.: Avian Salmonellosis, Its Economic and Public Health Significance. Official Rep. of Eighth World's Poult. Cong., (1948): 599-604.

[4] Hinshaw, W. R., McNeil, E., and Taylor, T. J.: Avian Salmonellosis, Types of Salmonella Isolated and Their Relation to Public Health. Am. J. Hyg., *40*, (1944): 264-278.

[5] Felsenfeld, O., Young, V. E., and Yoshimura, T.: A Survey of Salmonella Organisms in Market Meat, Eggs, and Milk. J.A.V.M.A., *89*, (1950): 65-76.

[6] Schnieder, M. D., and Gunderson, M. F.: Investigators Shed More Light on Salmonella Problem. U. S. Egg and Poult. Mag., *55*, (1949): 10-11, 22.

[7] Adler, H. E., Stadelman, W. J., and Holmes, M.: Unpublished data.

[8] Lee, C. D., Holm, G., and Murray, C.: Paratyphoid Infection in Turkeys. J.A.V.M.A., *89*, (1936): 65-76.

[9] Gauger, H. C., and Greaves, R. E.: Bacteriological

Examination of Shells and Contents of Eggs Laid by Turkeys Naturally or Artificially Infected with *S. typhimurium*. Poult. Sci., *25*, (1946): 119-123.

[10] Gauger, H. C., and Greaves, R. E.: Isolating of *Salmonella typhimurium* from the Feces of Turkeys. Poult. Sci., *25*, (1946): 232-235.

[11] Gauger, H. C., and Greaves, R. E.: Isolating of *Salmonella typhimurium* from Drinking Water in an Infected Environment. Poult. Sci., *25*, (1946): 476-478.

[12] Gauger, H. E., and Greaves, R. E.: Isolation of *Salmonella typhimurium* from the Feces of Artificially Infected Poults. Poult. Sci., *26*, (1947): 48-51.

[13] Pomeroy, B. S., and Fenstermacher, R.: Paratyphoid Infection of Turkeys. Am. J. Vet. Res., *2*, (1941): 285-291.

[14] Goresline, H. E., Howe, M. A., Baush, E. R., and Gunderson, M. F.: Inplant Chlorination Does a 3-way Job. U. S. Egg and Poult. Mag., *57*, (1951): 1-5.

Studies in Avian Neoplasia. III. Incidence of Rous Virus-Neutralizing Antibodies and Lymphomatosis in Chickens Inoculated with Neoplastic and Normal Tissue Suspensions

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THE PRESENCE of Rous-neutralizing antibodies (RNA) in many of the chickens from certain populations which experienced extensive losses due to lymphomatosis was observed by Duran-Reynals, according to Cottral and Winton.[4] Further reference was made to these studies by Winton [14] and by Duran-Reynals and Bryan.[10] A study of field flocks with marked losses due to lymphomatosis, by Kenzy and Neuzil,[13] indicated that RNA were present in over 60 per cent of the surviving birds. Chickens inoculated intravenously with lymphoid tumor extracts developed RNA in their serums.[10, 14]

The antigenic components in the lymphoid tumor extracts responsible for stimulating the formation of RNA were found to be destroyed by formalin according to Duran-Reynals and Bryan [10] who referred to Cottral's report.

In this study, chicks and adult birds were inoculated with either neoplastic or with normal tissue suspensions with the hope of clarifying the relationship of RNA and lymphomatosis.

MATERIALS AND METHODS

The Rous virus-neutralization (RVN) test as described by Duran-Reynals[7, 8] and modified by Kenzy [12] was used to determine the presence of RNA.

The Preparation and Inoculation of Tissue Suspensions.—The neoplastic and normal tissues were collected aseptically and minced in a cold (4 C.) Waring blendor with sufficient cold (4 C.) KCN-saline (as prepared by Dmochowski [6]) to give a 10 per cent suspension (on the basis of tumor weight per volume of diluent).

After filtering the tumor suspension through sterile gauze, 0.5 to 1.0 ml. was inoculated by either the intramuscular or intraperitoneal routes into White Leghorn chicks less than 4 weeks of age. In the case of tumors 363-1 and 741, both routes of inoculation were used. Tumor suspensions were sometimes stored in a dry ice chest at —70 C. until the time of inoculation, otherwise about 2 hours elapsed from the time of harvest to that of inoculation.

White Leghorn chickens 10 to 12 months of age were inoculated intramuscularly with 1 ml. of the various tissue suspensions each week for seven weeks. The first inoculation was made with the fresh tissue suspension while the others were made with material preserved by addition of 0.1 per cent formalin * (40 per cent formaldehyde) and refrigerated at 4 C.

Serums were collected for the RVN test from the adult chickens fifteen days after the last inoculation. The young birds, which were inoculated as chicks (less than 4 weeks old), were bled at about one, six, and ten months after inoculation. Controls and those inoculated were observed for one to two years.

The Source and Care of Experimental Birds.—All chicks used in these tumor studies were hatched from a closed inbred White Leghorn flock of 500 hens maintained in a central laying house at the Veterinary Research Institute. This laying house was quarantined and all service men were furnished with clean coveralls, caps, and boots before entering the chicken pens. The incidence of spontaneous lymphomatosis in this stock flock, which had been studied for several years, was observed to be 3.8 per cent during the two-year period of this study (1949-1950). All dead chickens were autopsied and microscopic tissue examinations carried out, if needed, to establish a diagnosis. Chicks from the same hatches as the inoculated chicks, and all chicks for the laying stock, were raised on wire ** in electrically heated brooders in a building completely isolated from the laying stock. At the age of 8 to 10 weeks, the

From the Veterinary Research Institute, Ames, Iowa. Some of the material in this report was taken from a thesis submitted to the Graduate School of Iowa State College in partial fulfillment of the requirements for the degree of doctor of philosophy. The coöperation of the Veterinary Research Institute staff is gratefully acknowledged.

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* At the time this study was initiated, the information concerning the effect of formalin on the antigenicity of tumor suspensions, as observed by Cottral, was not available.

** 801A Brooder, Oakes Manufacturing Co., Tipton, Ind.

young birds were placed on litter in the central laying house or in an isolated building. In certain of these experiments, the control chicks (less than 4 weeks old) were placed in isolation at the same time that the experimental chicks were inoculated.

Isolated chickens were maintained in separate buildings about 100 yards from any other chickens or in a separate room with an individual door to the outside. Caretakers always wore laundered coveralls and caps, which had not been used in any other poultry building. Boots or rubbers were scrubbed with a detergent-lye solution or with 3 to 5 per cent cresol (U.S.P.) quite thoroughly before entering the pens. Separate equipment for cleaning, feeding, and watering was kept with each pen.

Adult chickens, negative to the RVN test, were used in this study. Such birds were obtained from the source flock in the central laying house.

Pathology.—Tissues for microscopic examination were collected at the time of autopsy. Routine staining was carried out with Delafield's hematoxylin eosin. The identification and classification of neoplastic changes were based on Feldman and Olson's [1] discussion of avian neoplastic diseases.

Tumor Suspensions Used for the Inoculation of Chicks.—(1) Tumor 363-1 † was a myxosarcoma obtained from a 9-week-old New Hampshire Red pullet of a flock which experienced significant losses due to visceral lymphomatosis (VL). Five 3-week-old White Leghorn chicks were inoculated with this tumor and raised in isolation, while 80 controls from the same hatch were maintained in the central laying house.

2) Lymphoid tumor 363-2 (liver suspension) was obtained from a 5-month-old chicken with VL, which had been inoculated with myxosarcoma 363-1. This chicken had at least 26,000 Rous-neutralizing doses (RND) per milliliter of serum. Five 3-day-old chicks were inoculated with 363-2 and raised in isolation, while 70 controls from the same hatch were maintained in the central laying house.

3) Lymphoid tumor 363-3 (liver suspension) was obtained from a 3-month-old White Leghorn chicken with VL which had been inoculated with tumor 363-2. This bird had at least 500 RND per milliliter of serum. Six 1-week-old chicks were inoculated with 363-3 and raised in isolation, while 80 controls of the same hatch were maintained in the central laying house.

4) Lymphoid tumor 363-4 (liver suspension) was obtained from a 4-month-old White Leghorn chicken with VL and no demonstrable RNA. This chicken had been inoculated with tumor 363-2. Twenty-two 10-day-old chicks were inoculated and raised in isolation, while 150 controls of the same hatch were maintained in the central laying house.

5) Inoculum 546 consisted of a bone marrow suspension obtained from a 7-month-old White Leghorn chicken which survived the inoculation of myxosarcoma 363-1. Serum samples from this bird demonstrated at least 130,000 RND per milliliter in October, 1950, but no RNA were found two months later at the time it was killed.‡ Six 1-week-old chicks were inoculated with 546 and raised in a separate pen of the same building with 6 controls from the same hatch.

† This tumor and the respective flock history was made available by Dr. W. P. Switzer, Veterinary Research Institute, who was then in the Iowa Veterinary Diagnostic Laboratory.

‡ The feet of this bird were severely injured due to freezing, which may have affected the euglobulin level.

TABLE 1—Incidence of Rous-Neutralizing Antibodies and Lymphomatosis in Birds Inoculated with Normal* and Neoplastic Tissue Suspensions

Suspension (No.)	Inoculated				Controls			
	Per cent with RNA**		Per cent with lymphomatosis		Per cent with RNA		Per cent with lymphomatosis	
310	0	0/23†	8	2/23	0	0/20	25	5/20[a]
363-1	80	4/5	60	3/5	10	15/153[b]	3.8[b]	
363-2	40	2/5	60	3/5	10	15/153[b]	3.8[b]	
363-3	16	1/6	83	5/6	10	15/153[b]	3.8[b]	
363-4	10	2/20	78	18/23	10	15/153[b]	3.8[b]	
367	0	0/23	13	3/23	0	0/20	25	5/20[a]
403	0	0/9	3	3/83	0	0/10	3	3/80[c]
546	20	1/5	16	1/6	20	1/5	20	1/5 [d]
636	5	1/19	26	6/23	0	0/16	17	4/23[e]
641	0	0/17	8	2/23	0	0/16	17	4/23[e]
741	25	3/12	57	8/14	....	.....	0	0/30[f]

* No gross or microscopic evidence of lymphomatosis observed; ** serum with demonstrable neutralizing properties for the Rous virus; † numerator of fraction indicates the number of positive observations, while the denominator refers to the total number of birds observed.

[a] Controls and those inoculated for both these tumors were raised together in a separate building. [b] These controls were raised in the central laying house. The entire population in this building during the period 1949-1951 was considered in obtaining these figures. [c] Controls and those inoculated were raised in the same pen of an isolated building. [d] Controls and those inoculated were raised in different pens which were separated from other pens of young inoculated birds in the same building. [e] These controls were raised in a separate pen but in a building where other young inoculated birds were kept. [f] These controls were raised in a separate isolated building.

6) Tumor 741 ♀ was a myxosarcoma obtained from a 12-week-old New Hampshire Red pullet. Fourteen 12-day-old chicks were inoculated with 741 and raised in isolation, while 30 controls from the same hatch were raised in a separate isolated building.

7) Tumors 310, 367, and 403 were obtained from the affected livers of 10- to 12-month-old chickens with VL selected from a White Leghorn flock with 3.8 per cent loss due to this disease. No RNA were observed in these tumor donors; however, such antibodies were present in 10 per cent of 153 chickens tested from this laying flock during 1949 to 1951. The number of inoculated chickens and controls for each of these tumors is listed in table 1.

8) Tumor 636 was obtained from the liver of an 11-month-old chicken with VL which was selected from a New Hampshire Red flock that had experienced a 5 per cent loss due to this disease. No RNA were demonstrated in this flock. Twenty-three 2-week-old White Leghorn chicks were inoculated with 636.

9) Tumor 641 was obtained from the liver of an 8-month-old chicken * with VL, which was selected from a White Leghorn pen in which the incidence of this disease was 15 per cent. No RNA had been demonstrated during the two-year observation of this pen. Twenty-three 2-week-old chicks were inoculated with 641.

Materials Used for Inoculation of Adult Chickens.—(1) Tumor 310 was obtained from the affected liver of a 10-month-old chicken with VL, selected from a White Leghorn flock with a 3.8 per cent loss due to this disease. No RNA were observed in this tumor donor.

2) Tumor 378 was obtained from the affected nerves of 4, 5-week-old birds with neural lymphomatosis, selected from a New Hampshire Red flock with 5 per cent lymphomatosis and no history of demonstrable RNA.

3) Tumor 453 was obtained from the affected nerves of a 6-month-old chicken with neural lymphomatosis, selected from a White Leghorn flock with 3.8 per cent lymphomatosis and a history of RNA in about 10.0 per cent of the chickens tested.

4) Tumor 454 was obtained from the liver of a 6-month-old White Leghorn chicken with VL, selected from a flock with a 30 per cent loss due to this disease. High levels of RNA were present in 2 of the chickens tested (at least 37,600 RND/ml. of serum).

5) Inoculum 460 was a suspension of the normal liver and sciatic nerves of a 6-month-old White Leghorn chicken from a flock with 3.8 per cent loss due to lymphomatosis and a 10.0 per cent incidence of RNA.

6) Tumor 473 (liver suspension) was obtained from a 5-month-old White Leghorn chicken with VL and at least 26,000 RND per milliliter of serum, which had been inoculated with myxosarcoma 363-1.

7) Tumor 477 was obtained from the liver of a 10-month-old White Leghorn chicken with VL. The source flock had 3.8 per cent loss due to lymphomatosis and a 10.0 per cent incidence of RNA in 153 birds tested during 1949-1951.

8) Tumor 479 was obtained from the liver of an 8-month-old chicken (New Hampshire Red-White Leghorn cross) with VL and at least 5,200 RND per milliliter of serum. The source flock had experienced a 37 per cent loss due to lymphomatosis and RNA were observed in 60 per cent of the survivors.

RESULTS AND DISCUSSION

A statistical analysis ** of the data in table 1 indicates that the percentage occurrences of VL in the inoculated groups of 363-1, 363-2, 363-3, 363-4, and 741 are all statistically significant ($P < 0.01$) when each is tested singly against the control.

The data in table 1 indicate that RNA were present in 80 per cent of the chickens inoculated with myxosarcoma 363-1 and in 40 per cent of those inoculated with the lymphoid tumor 363-2 but only in an occasional chicken inoculated with the lymphoid tumors 363-3 and 363-4. At the same time, the incidence of lymphomatosis varied from 60 to 83 per cent. Possibly the agent stimulating RNA became inactive or was lost during these serial passages. Carr[3] has reported the presence of latent Rous virus in a host for over a year before neoplastic changes occurred. In another study at this laboratory (unpublished data), connective tissue tumors developed in 2 of 110 chickens inoculated with a lymphoid tumor obtained from a 4-month-old White Leghorn chicken with VL, which had been inoculated with 363-3, while the 200 controls were negative for such tumors.

Another, myxosarcoma 741, stimulated the formation of RNA in only 25 per cent of the inoculated chickens, whereas lymphomatosis developed in 57 per cent. Serial passage of the lymphoid tumor which developed was not attempted.

§ This tumor and the respective flock history were made available by Dr. L. D. Jones of the Iowa Veterinary Diagnostic Laboratory.

* This chicken had been inoculated with tumor 367 suspension about eight months earlier (table 1).

** Dr. A. W. Nordskog, Poultry Department, Iowa State College, Ames, carried out the statistical analysis of the data in table 1, based on *chi* square test as outlined by Fisher and Yates in Statistical Tables for Biological, Agricultural, and Medical Research. 1949: 46 (table 8). Oliver and Boyd, Edinburgh.

On the basis of our previous experience, the Rous sarcoma must apparently develop and begin to regress before definite levels of neutralizing antibodies are demonstrated. It is interesting, therefore, to find these antibodies in 80 per cent of the birds inoculated with the myxosarcoma 363-1, although evidence of connective tissue tumor development was observed in only 1 chicken. Likewise, in the case of the myxosarcoma 741, only 1 of the chickens developed a connective tissue tumor, although RNA were demonstrated in 25 per cent of those inoculated. The presence of RNA in the chickens inoculated with myxosarcomas 363-1 and 741 would suggest an antigenic relationship between the agents in these two tumors and the Rous virus. Andrewes [1] has shown that some of the connective tissue tumor agents were antigenically related.

Definite evidence of a connective tissue tumor agent or a lymphoid tumor agent was not observed in the chickens inoculated with the suspension 546 (table 1), although this donor had been inoculated with myxosarcoma 363-1 and had exhibited a high level of RNA at one time.

The inoculation of the lymphoid tumors 310, 367, 403, 636, and 641 (table 1) failed to produce a definite increase in the incidence of RNA or lymphomatosis. In fact, some of the controls exhibited more lymphomatosis than those inoculated; however, such differences were not considered of importance. All of these lymphoid tumors were collected from flocks which were experiencing a relatively low incidence of this disease (3 to 5 %); thus, a masked agent or one of very low virulence might be present. In view of the lack of transplantability of these tumors, the suggestion that they be classified as lymphocytomas should be considered (Feldman and Olson [11]). The review of Davis and Doyle [5] emphasized the different results obtained by various workers in studying the transmission of lymphoid tumors. More recently, Burmester and Denington [2] have reported variations in the transmissibility of tumor extracts from different spontaneous cases of lymphomatosis.

A few chickens inoculated with normal and tumor tissue suspensions developed RNA (table 2). Possibly more of those inoculated would have developed RNA if formolized suspensions had not been used for most of the inoculation series. The presence of RNA in 1 of the 2 chickens inoculated with the normal tissue suspension 460 may suggest that a latent agent was present in the donor (table 2). However, since the chickens were kept in individual cages in the same room, there was also the possibility of air transmission of a connective tissue virus. Duran-Reynals [9] has observed a many-fold increase of RNA in birds kept in the same room with chickens and ducks supporting lymphoid and connective tissue tumors. We have observed contact transmission of a connective tissue tumor agent based on the occurrence of RNA in chickens which were hatched and raised with other chickens from a contaminated population (unpublished data).

TABLE 2—Incidence of RNA* in Adult Chickens Inoculated with Normal and Neoplastic Tissue Suspensions

Suspension (No.)	Number of Rous (+) birds[a]	Number of Rous (−) birds[b]
310[c]	1	2
378[c]	0	2
453[c]	0	2
454[c]	1	3
460[d]	1	1
473[c]	0	2
477[c]	0	1
479[c]	0	2

* Rous neutralizing antibodies.
[a] Chickens with demonstrable neutralizing properties for the Rous virus present in the serum. [b] Chickens without demonstrable neutralizing properties for the Rous virus present in the serum. [c] Lymphoid tumor suspensions. [d] Normal tissue suspension.

SUMMARY

1) The presence of Rous-neutralizing antibodies (RNA) was demonstrated in 4 of the 5 chickens inoculated with myxosarcoma 363-1. Three of these 5 chickens died of lymphomatosis. Serial subinoculation of lymphoid tumor 363-2, which developed in a chicken inoculated with 363-1, produced 60, 78, and 83 per cent visceral lymphomatosis (VL), respectively, in the second, third, and fourth inoculation series, whereas the corresponding incidence of RNA was 40, 16, and 10 per cent.

2) Only 25 per cent of the serums from the 14 birds inoculated with the myxosarcoma 741 demonstrated RNA, whereas 57 per cent of these chickens died of VL.

3) Transplantation attempts of five different lymphoid tumors in young chicks

were not successful in increasing the incidence of lymphomatosis or RNA.

4) Inoculation of seven different lymphoid tumor suspensions into adult birds failed to definitely increase the incidence of RNA.

References

[1] Andrewes, C. H.: Further Serological Studies on Fowl-Tumor Viruses. J. Path. and Bact., *37*, (1933): 27-44.

[2] Burmester, B. R., and Denington, E. M.: Studies on the Transmission of Avian Visceral Lymphomatosis. I. Variation in Transmissibility of Naturally Occurring Cases. Cancer Res., *7*, (1947): 779-785.

[3] Carr, J. G.: Observations upon Spontaneously Recurring Rous No. 1 Tumors. Brit. J. Exptl. Path., *23*, (1942): 206-213.

[4] Cottral, G. E., and Winton, B.: Conference on Fowl Lymphomatosis. Regional Poultry Research Laboratory, East Lansing, Mich. 1948. (Personal communication.)

[5] Davis, O. S., and Doyle, L. P.: Studies in Avian Leukosis. I. The Transmissibility of Visceral Lymphomatosis. Am. J. Vet. Res., *8*, (1947): 103-112.

[6] Dmochowski, L.: Investigations on the Properties of Agents Causing Fowl Tumors. I. Attempts at Isolation of the Fowl-Tumor Agents by Differential Centrifugation. J. Nat. Cancer Inst., *9*, (1948): 57-67.

[7] Duran-Reynals, F.: Neutralization of Tumor Viruses by the Blood of Normal Fowls of Different Ages. Yale J. Biol. and Med., *13*, (1940): 61-76.

[8] Duran-Reynals, F.: Yale University School of Medicine, New Haven, Conn.: Information Concerning the Technique of the Rous Serum Neutralization Test (personal communication). 1949.

[9] Duran-Reynals, F.: Neoplastic Infection and Cancer. Am. J. Med., *8*, (1950): 490-511.

[10] Duran-Reynals, F., and Bryan, E.: Studies on the Origin of the Naturally Occurring Antibodies Against Tumor Viruses Developing in Aging Chickens. (To be published.)

[11] Feldman, W. H., and Olson, C., Jr.: Neoplastic Diseases of the Chicken. *In:* Biester, H. E., and Schwarte, L. H.: Diseases of Poultry. 2nd ed. The Iowa State College Press, Ames, Iowa, (1948): 637-714.

[12] Kenzy, S. G.: Studies in Avian Neoplasia. I. A Quantitative Evaluation of Neutralizing Antibodies for the Rous Sarcoma Virus in Avian Serums. Am. J. Vet. Res., *13*, (1952): 388-394.

[13] Kenzy, S. G., and Neuzil, P. V.: Studies in Avian Neoplasia. II. Incidence of Rous Virus Neutralizing Antibodies in Serums Collected from Flocks Experiencing Losses Due to Lymphomatosis. Am. J. Vet. Res., *14*, (1953): 123-128.

[14] Winton, B.: Eleventh Annual Report of the Regional Poultry Research Laboratory. East Lansing, Mich. 1950.

The Blood and Urine Findings in Infectious Canine Hepatitis

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IT HAS been shown, by means of the complement-fixation test, that infectious canine hepatitis exists throughout the world in the canine population in much greater frequency than is reflected by cases seen at autopsy.[1] An urgent problem, therefore, is the diagnosis of the disease by clinical means. From observations at the Angell Memorial Animal Hospital, it appears that all gradations of the infection exist, from subclinical to the most peracute, overwhelming type.

The clinical problems, therefore, appear to be: (1) primary recognition of the disease; (2) differentiation from other canine infectious and noninfectious disorders which might produce symptoms of infectious disease or collapse; (3) differentiation between the cases which are likely to terminate fatally and those that will recover; (4) determination of the stage of the illness at the time of the first examination, so that appropriate supportive treatment may be instituted.

Clinical laboratory procedures have been useful at the Angell Memorial Animal Hospital in supplying data contributing to steps 1 and 2 above and, if inferences may be drawn from experimentally induced cases, useful information regarding steps 3 and 4 should also be forthcoming from careful clinical laboratory work.

Studies are still continuing, but it would appear that sufficient progress has been made to report results on the clinical cases at the Angell Memorial Animal Hospital and on the experimental laboratory investigation carried on in conjunction with Dr. Victor J. Cabasso.

CLINICAL STUDIES

Because of the short course and sudden, fatal termination of many of our cases of infectious canine hepatitis, blood and urine studies have been completed on but 22 of the 66 fatal cases previously reported.[2] The results of this work are summarized in table 1.

The blood picture of the fatal cases was characterized by a reduction in the total leukocyte count, variable sedimentation rate, normal or increased hemoglobin content, and normal urea nitrogen values. Urinalysis revealed concentration, albuminuria and, frequently, bilirubinuria.

Clinical laboratory examinations have been performed on 100 cases, fitting the diagnostic criteria of hepatitis[3] which did not succumb. In general, these cases were admitted with a moderate or high fever, depression, swelling and reddening of the tonsils and, occasionally, enlarged lymph nodes. They differed in no special way from the febrile animals among the fatal cases, except that death did not ensue. Respiratory symptoms were minimal and nephritis was not suspected. These cases did not subsequently develop symptoms of distemper or leptospirosis. A fair number of them developed corneal opacity during the convalescent stage, which appears characteristic of canine hepatitis. These are summarized in table 2.

TABLE 1—Tabulation of the Blood and Urine Data from 22 Fatal Cases of Infectious Canine Hepatitis, Showing the Mean and the Range of the Various Elements

	Examined (No.)	Mean	Lowest	Highest
		BLOOD		
Hemoglobin	8	12.3	10.0	20.8
Leukocytes	12	6,070	1,550	14,900
Sediment. rate	7	6/30, 17/60	0/30, 1/60	25/30, 51/60
Urea nitrogen	2	12	18	20
		URINE		
Specific gravity	11	1.034	1.025	1.042
Albumin	11	+++	–	++++
Bilirubin	11	+++	–	++++

Hemoglobin in grams per 100 cc.; leukocytes in thousands; sedimentation rate in millimeters of fall for thirty and sixty minutes; urea nitrogen in milligrams per 100 cc. of whole blood; albumin and bilirubin by the qualitative test.

EXPERIMENTAL STUDIES

Opportunity was afforded to observe the blood picture of 7 puppies experimentally infected with infectious canine hepatitis.* These consisted of two litters of distemper-immune Basenjis, 4 and 5 months of age, raised under controlled conditions. The animals were observed for seven days prior to

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* These investigations were carried on at Lederle Laboratories, Pearl River, N. Y., on experimental animals used in the Virus Research Section.

TABLE 2—Tabulation of the Blood and Urine Data on 100 Nonfatal Cases Diagnosed Clinically as Infectious Canine Hepatitis, Showing the Mean and Range of the Various Elements.

	Examined (No.)	Mean	Lowest	Highest
BLOOD				
Hemoglobin	63	12.2	7.0	15.4
Leukocytes	95	10,600	3,400	23,900
Sediment. rate	65	12/30, 29/60	0/30, 0/60	46/30, 58/60
Urea nitrogen	4	14.2	11.0	20.0
Sugar	9	82	68	94
URINE				
Specific gravity	15	1.023	1.016	1.040
Albumin	11	+	+	+++
Bilirubin	8	++	±	+++

Hemoglobin in grams per 100 cc.; leukocytes in thousands; sedimentation rate in millimeters of fall for thirty and sixty minutes; urea nitrogen and sugar in milligrams per 100 cc. of whole blood; albumin and bilirubin by the qualitative test.

inoculation, during which period they appeared normal. The anterior chamber of the right eye of the 7 puppies was inoculated with infective liver suspension and the left anterior chamber with normal liver suspension. Blood samples were taken each day into ammonium and potassium oxalate bottles for sedimentation rates and white blood cell counts. Blood smears were prepared on slides directly from the whole blood at the time the samples were collected. Coagulation times were run by the capillary tube method. Leukocyte counts were calculated on the average of two counts from the same pipette. The sedimentation rates were determined in Wintrobe hematocrit tubes and clocked at thirty and sixty minutes. Hematocrits were packed at 3,200 r.p.m. for thirty minutes in a size 1 SB International centrifuge (diameter of head 10¼ in.). This method was shown to be reproducible by trial centrifugation.

The experiment, planned to obtain material for another purpose,[4] was conducted as follows: 7 dogs were inoculated; 1 dog was destroyed each succeeding day, after the onset of symptoms was noted. This order was maintained except where spontaneous death of a pup intervened. Consequently, the number of pups decreased as the experiment progressed. This situation was not ideal for hematological research but was necessitated by the plan of the experiment. Unfortunately, not enough animals were at hand to allow controls for the hematological portion of the experiment.

Experimental Results

The leukocyte level, which had fluctuated widely prior to inoculation, increased slightly on the second postinoculation day but did not exceed the high point recorded before inoculation. This was succeeded by a very rapid decline on the third postinoculation day. This trend was continued until the fifth day, at which time the first spontaneous death occurred. A slight upward trend was noted on the sixth day and continued as the number of dogs decreased (table 3). One dog was destroyed in extremis on the sixth day. The precipitous decline in leukocytes is not only reflected by the mean figures of table 3 but also shows a strikingly parallel course among the animals of the group due to uniformity of onset.

There was remarkable similarity in the progression of the changes in the sedimentation rate and leukocyte concentration during the early and middle course of the disease (fig. 1). The sedimentation rate increased significantly by the second postinoculation day and became progressively more rapid as long as the experiment continued. The apparent decrease of the sedimentation rate on the fifth day, noticeable in the average figures, was produced by a precipitous slowing of the rate of the dogs in extremis. This will be commented on later. A similar trend occurred in the mean

TABLE 3—Leukocyte Counts on 7 Individual Dogs Experimentally Infected with Infectious Canine Hepatitis

Dog (No.)	Days Pre- or Postinfection													
	−7	−6	−5	−4	−3	0	+1	+2	+3	+4	+5	+6	+7	+8
50	14.7*	24.6	18.8	24.2	27.0	20.9	17.3	21.7						
52	16.3	22.6	19.5	20.9	20.7	17.3	16.6	19.5	8.2					
51	14.9	21.0	22.5	25.8	25.9	20.6	22.6	28.6	11.2	5.5				
55	31.8	28.8	36.7	37.4	38.6	28.9	25.8	33.5	17.7	11.9	3.5			
53	16.8	31.7	30.2	33.7	22.9	20.1	18.7	28.5	9.7	9.0	4.7	5.5		
56	23.7	23.3	30.0	22.9	34.4	32.0	27.5	34.8	13.4	10.7	3.4	4.5	5.0	
54	10.3	26.0	26.0	32.4	32.1	27.0	32.6	29.1	9.2	8.7	5.8	7.5	7.5	14.9
Arithmetic mean	18.3	25.4	26.2	28.2	28.8	23.8	23.0	27.9	11.5	9.1	4.3	5.8	6.2	14.9

* Leukocyte count expressed in thousands. These counts seem rather high for puppies but are typical for those kept in this environment. The pups appeared normal in all other respects.

temperature curve produced by subnormal temperatures on the same day. The hematocrit showed a small but definite downward trend throughout the course of the experiment from a mean value of 42 per cent at the beginning to 29 per cent at the termination. Figure 2 shows this gradual downward trend in hematocrit in pup 54, destroyed on the eighth postinoculation day. This figure also demonstrates the continued acceleration of the sedimentation rate, even though the leukocytes were returning toward the normal figure and the body temperature was within normal limits for a pup. This tendency was also present in convalescent dogs among our clinical material. At this time, the animal appeared clinically normal and was classed as being in the convalescent stage.

The differential picture showed a reduction in all classes of leukocytes as the disease progressed and the general course was not unlike that of panleukopenia of cats.[5] There was at first a slight neutrophilia with an overall increase in both segmented and nonsegmented neutrophils, which appears rather impressive when computed on the basis of percentage but is seen to be only fleeting when the differential count is expressed by numerical values (fig. 3).

The reduction in the number of neutrophils was most marked, but the number of all other types of leukocytes was also reduced. Smith[6] reported a somewhat similar picture but attributed much of the leukopenia to a reduction of the concentration of lymphocytes. Our data do not confirm this impression. Eosinophils (not plotted in fig. 3) tended to disappear from the blood entirely at the point of maximum leukopenia.

The fact that the sedimentation rate slowed prior to death among the dogs in our clinical material has been mentioned earlier. This same phenomenon occurred in pup 51, which was killed in extremis (sedimentation rate: 2/30 and 8/60, hematocrit 17), and in pup 55 which died spontaneously (sedimentation rate: 1/30 and 3/60, hematocrit 10). Figure 4 illustrates this course in pup 55. Icterus index values remained normal in all the dogs save pup 55, in which an icterus index of 10 units was found just prior to death (normal 1 to 6). Clotting was delayed, time varying from fifteen to twenty minutes or more by the capillary tube method (normal up to six minutes). One blood specimen failed to clot in a flask after eighteen hours of storage. Clot contractility was likewise poor,

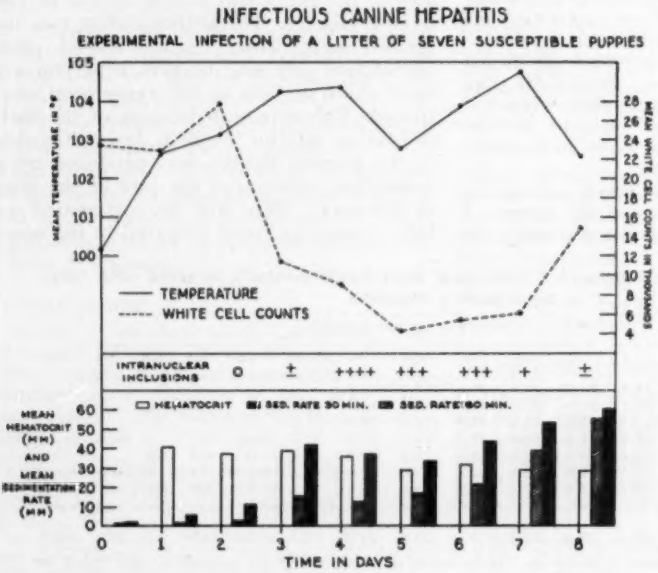
INFECTIOUS CANINE HEPATITIS
EXPERIMENTAL INFECTION OF A LITTER OF SEVEN SUSCEPTIBLE PUPPIES
MEAN TEMPERATURE IN °F
MEAN WHITE CELL COUNTS IN THOUSANDS
TEMPERATURE
WHITE CELL COUNTS
INTRANUCLEAR INCLUSIONS
0 ± ++++ +++ +++ + ±
HEMATOCRIT
SED. RATE 30 MIN.
SED. RATE 60 MIN.
MEAN HEMATOCRIT (MM) AND MEAN SEDIMENTATION RATE (MM)
TIME IN DAYS

Fig. 1—Graphs for total leukocytes, hematocrit, sedimentation rate, and body temperature plotted from the mean values from 7 dogs. Note the initial elevation of leukocyte figure on the second postinoculation day (this may or may not be significant, since it did not rise above the highest preinoculation point), followed by a precipitous fall coinciding with clinical crisis and a later rise in value concomitant with beginning recovery. The hematocrit values fell throughout the course of the disease, reflecting the depressed erythropoiesis. Note also the points of appearance and disappearance of intranuclear inclusions in liver cells.

suggesting a deficiency in blood platelets. This impression was borne out by qualitative estimation on stained blood smears, where platelets appeared markedly reduced during the period of crisis. Limited studies of bone marrow showed marked depression of erythropoiesis, leukopoiesis, and thrombocytopoieses at the time of crisis with a rapid regeneration thereafter.

DISCUSSION

In comparing the laboratory data from the fatal and nonfatal cases in the clinical material, one notices agreement, generally, in the hemoglobin values. The higher mean leukocyte concentration in the nonfatal cases is to be expected because of less severe affection with a virus disease. The gross data for sedimentation rate, however, show some difference in that the rates tend to be higher in the nonfatal cases. This is especially notable when the 2 cases are removed in which the test was run two days prior to death, namely, 6/30, 35/60 and 25/30, 51/60. It will be seen that the other fatal cases exhibit a very low sedimentation rate. Thus, the hypothesis that the difference was brought about by approaching death appeared possible. In other words, a slowed sedimentation rate was indicative of an unfavorable prognosis. A probable explanation for the phenomenon, advanced from the evidence at hand, was that approaching death was heralded by peripheral circulatory collapse with consequent hemoconcentration and slowing of the sedimentation rate. (It is known that other factors being equal, the sedimentation rate is inversely proportional to the erythrocyte concentration.) However, such a theory was refuted when the same phenomenon was observed in the experimentally induced cases where it occurred concomitantly with a rapid reduction in hematocrit in contradistinction to the hemoconcentration noted in the fatalities at the hospital. It is patent, therefore, that the reduction in sedimentation rate is significant, and probably relates to change in the plasma proteins and is not merely an artifact relating to changes in hematocrit. No time was available for plasma-protein studies to be carried out in these animals. Somewhat supporting the above hypothesis was the increased clotting time (possibly due to terminal fibrinolysis or an increase in antithrombin) which was observed during this period.

SUMMARY AND CONCLUSIONS

Clinical laboratory studies were performed on 22 cases of hepatitis, which later

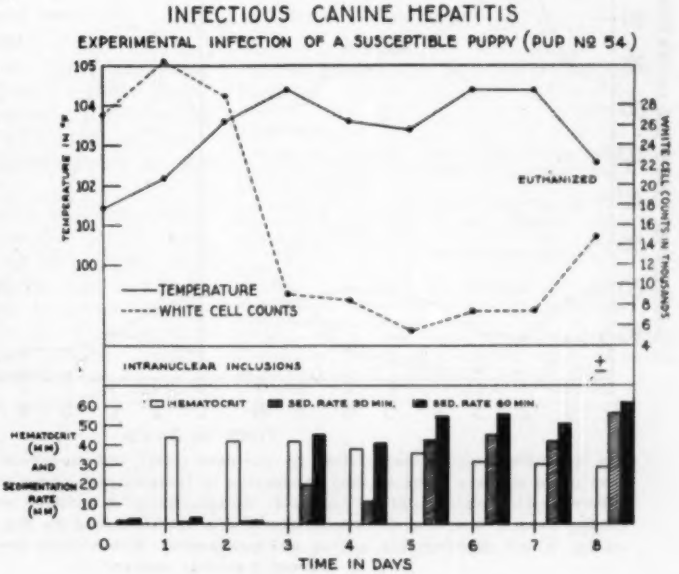

Fig. 2—Values for hematocrit, total leukocytes, sedimentation rate, and body temperatures from pup 54, destroyed eight days after inoculation when appearing clinically normal. Note the downward trend of the hematocrit. The leukocytes appear to be returning toward the normal level, while the sedimentation rate is still accelerating. Note the reduction of intranuclear inclusions in liver cells.

died and were confirmed by histopathological means. The blood picture was characterized by leukopenia, (mean w.b.c.—6,070; lowest—1,500, highest—14,900), variable sedimentation rate (mean—6/30 and 17/60; lowest—0/30 and 1/60, highest—25/30 and 51/60), slightly reduced, normal, or increased values for hemoglobin, and normal values for urea nitrogen. The urine was characterized by high concentration (mean specific gravity—1.034; lowest—1.025, highest—1.042), albuminuria, and bilirubinuria. The sedimentation rates tended to be increased on those animals in which the sample was taken one or more days prior to death and normal or slowed on those in which the sample was taken on the terminal day.

There is good correlation between the findings in the fatal cases and those obtained during the period of crisis in the experimental cases. The slowing of the sedimentation rate, noted clinically, is borne out in the experimental disease and appears of prognostic value. Further study of this and related phenomena is indicated to serve as a basis for treatment and to shed light on the cause of the terminal hemorrhage and the mechanics of death.

Laboratory data are available on 100 cases clinically diagnosed as infectious canine hepatitis, which later recovered. These cases were not confirmed by histopathological or virological means and certainly are not completely homogeneous. It is felt, however, that most were cases of infectious hepatitis. The blood picture was characterized by leukopenia and increased sedimentation rate. The leukocyte concentration was (mean—10,600; lowest—3,400, highest—23,900). The sedimentation rates were (mean—12/30 and 29/60; lowest—0/30 and 0/60, highest 46/30 and 58/60). The urine was of normal density (mean specific gravity—1.023; lowest—1.016, highest—1.040) and contained a trace-to-plus four albumin. Urinary bilirubin was increased. Sugar and urea nitrogen levels were within normal limits.

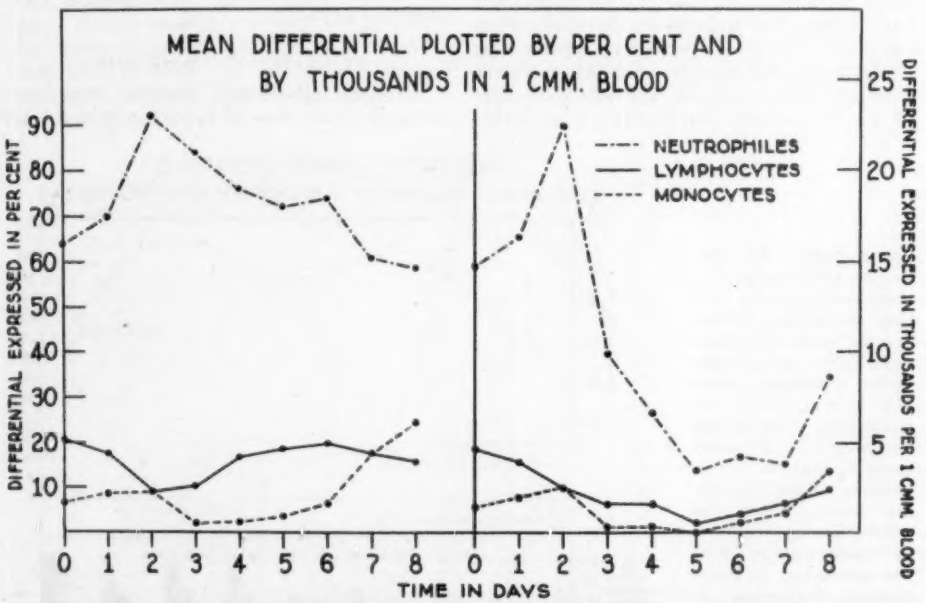

Fig. 3—Differential values plotted by per cent (left) and numerical values (right). Note that there appears to be an early reduction in lymphocytes (days 2 and 3) followed by a return to the preinoculation per cent. Subsequently, a relative monopenia is apparent during days 3, 4, 5, and 6. These trends are not sustained by the curves for numerical values which demonstrate a true panleukopenia. Eosinophils are not plotted but followed a similar course.

Clinical laboratory studies were performed on 7 experimentally infected pups. The blood picture was characterized by a tion of platelets, pointing to a depression of the genesis of erythrocytes and platelets, as well as of leukocytes. The clotting time

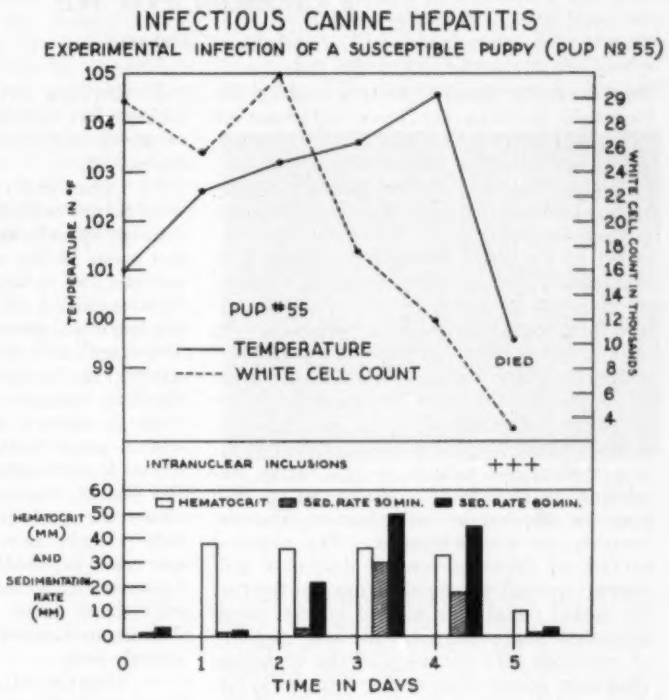

Fig. 4—Values for hematocrit, total leukocyte, sedimentation rate, and body temperature of pup 55 which died on the fifth postinoculation day. Note the sudden slowing of the sedimentation rate coincident with the fall in hematocrit (due to hemorrhage) which heralds approaching fatal termination. Note abundance of intranuclear inclusions in liver cells.

fleeting neutrophilia two days subsequent to inoculation, followed by a rapidly developing, progressive leukopenia (contributed to by a reductoin in all classes of leukocytes) reaching the lowest point at the time of clinical crisis.

The sedimentation rate was accelerated early and did not return to normal with the posterisis increase in leukocytes. In 2 cases, there was a sudden diminution in sedimentation rate prior to developing extremis. This appears to be of prognostic importance. There was a gradual diminution in hematocrit throughout the course of the disease, as well as a qualitative diminu- was prolonged and the clot retraction was poor.

References

[1] Rubarth, S.: An Acute Virus Disease with Liver Lesions in Dogs. Acta Path. et microbial. Scand., *89*, (1947): 1-222.

[2] Coffin, D. L.: The Rapid Diagnosis of Contagious Canine Hepatitis from Autopsy Material. J.A.V.M.A., *120*, (1952): 287-290.

[3] Coffin, D. L.: Los Infermedades infecciosos de los perros. In preparation.

[4] Coffin, D. L., Coons, A., and Cabasso, V. J.: A Study of Infectious Canine Hepatitis by the Fluorescent Antibody Technique. In preparation.

[5] Riser, W. H.: The Behavior of the Peripheral Blood Elements in Panleukopenia (Agranulocytosis) in the Domestic Cat. Am. J. Vet. Res., *8*, (1947): 82-90.

[6] Smith, D. L. T.: Observations on Infectious Canine Hepatitis. Am. J. Vet. Res., *12*, (1951): 38-43.

Intervertebral Disc Protrusions in the Dog
I. Incidence and Pathological Lesions

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THE INTERVERTEBRAL disc is a cushion interspaced between the bony vertebrae of the spinal column. Its function is to allow movement between the vertebrae, to help bind them together, and to absorb the traumatic shock occurring to the spinal column during normal living. This disc is composed of an outer laminated fibrous portion called the annulus fibrosus. Within the annulus is contained a semigelatinous-like fluid called the nucleus pulposus. On each vertebral border of the disc is the cartilaginous plate which, in turn, is cemented to the ends of the vertebrae by a thin layer of calcified cartilage.[15]

The pathological appearances of the intervertebral disc take on several forms. According to Donahue,[3] a degenerating disc may be affected by dehydration, fibrosis, necrosis, or a calcification. The exposed surface of this degenerated disc may still appear normal as viewed from the floor of the spinal canal and will, of course, cause no spinal compression. A disc may rupture or protrude into the body of the vertebrae (Schmorl lesion)[19] as well as ventrally, laterally, or dorsally. However, in this paper we are largely concerned with dorsal protrusions into the spinal canal. These may be classified as:

1) Herniation of the nucleus pulposus without rupture of the annulus fibrosus. In this case, the dorsal annulus has been weakened and a "bulge" has occurred in which the nucleus has protruded dorsally. However, it is still encased in the annulus fibrosus (fig. 1).

2) Rupture of the annulus in which the nucleus has extruded itself into the spinal canal through this break in the annulus fibers (fig. 2).

3) Occasionally in man, an edema of the disc occurs which will cause a projection into the spinal canal. Deucher [2] claims this can occur in the annulus as well as in the nucleus and is frequent in young patients. This condition, it is said, can occur when the normal forces keeping it in place are decreased and the nucleus swells from edema. A disc is said to increase up to 20 times its normal size when placed in water.

4) A rupture of the annulus fibrosus with a projection of the nucleus under the dorsal longitudinal ligament (fig. 3). Here the dorsal longitudinal ligament still encloses the nucleus but a "bulge" will result into the canal. In this regard, there are cases in which the dorsal longitudinal ligament hypertrophies, as a result probably from some unusual stress or senile change, and protrudes abnormally into the spinal canal.

5) Occasionally, a spondylitis deformans and related conditions may produce bony projections dorsally (osteophyte formation) as well as ventrally. This condition may or may not involve the disc but

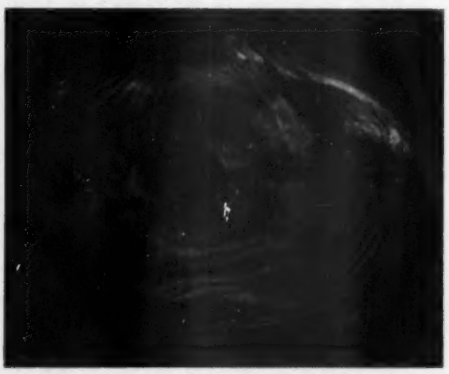


Fig. 1—A cross section of a herniated nucleus pulposus. The annulus fibrosus is still intact but is greatly weakened.

This paper is an excerpt from a thesis presented to the faculty of the Graduate School of Cornell University for the degree of doctor of philosophy in June, 1952.

The author expresses his appreciation to the Research Council of the American Veterinary Medical Association for the fellowship which has made this study possible.

The author also acknowledges the untiring help and consideration of his special committee—Dr. E. P. Leonard, Dr. H. H. Dukes, and Dr. Peter Olafsen; to the staff members of the following departments—Small Animal Disease and Therapeutics, Pathology, and Physiology; to Dr. John Bentinck-Smith for the bacteriological work performed; to the technical assistance of Mrs. Jeanne Baker; and to Mr. Edmund LaValle in preparing tissue sections.

of course can cause damage to nervous tissue.[16]

The conditions listed above (except for spondylitis) will be referred to collectively as "protrusions of the intervertebral disc." It is felt that the "enchondrosis intervertebralis"[15] is partially outmoded, since this term implies that the protruding mass is a cartilaginous growth or tumor instead of misplaced disc tissue. However, the term does contain some truth in that, although this condition is due to a ruptured disc, it is also, according to Hansen,[7] a chondroid metaplasia of the nucleus.

INCIDENCE

It is difficult to state the true clinical incidence of disc protrusion in the dog. Diagnoses which may often be confused with disc disease might be listed as arthritis, rheumatism, traumatic bruises, nephritis, fractures of the vertebrae, enteritis, spondylitis, and various other nervous disorders.

At the small animal clinic of the New York State Veterinary College, during a period of twelve months (September, 1950, to September, 1951), 5,154 patients were admitted. Of this number, 1,690 were cats and animals other than dogs. During this period, 25 cases of disc protrusion nervous disorders were seen in dogs for an incidence of 0.7 per cent (25÷3464 dogs). One might then say that, in the ordinary small animal practice, 0.5 to 1.0 per cent of the total number of animals seen will suffer from this condition.

In regard to over-all incidence, the author pondered the question as to how many dogs have disc protrusions and do not show marked clinical symptoms. A survey was conducted on routine canine autopsies where the animals had died or had been destroyed for a condition other than disc protrusion. The spinal canal floor was exposed by carefully removing the dorsal laminae. The disc surfaces were then observed for protrusion. It was found that out of 130 dogs, 41.5 per cent showed disc protrusions. Eighty-seven per cent of these positive dogs were over 5 years of age, while 63 per cent of all the dogs 5 years and older had positive lesions. Seventy-six per cent of the positive dogs had more than one disc lesion. Thirty males and 24 females were affected. Twenty-two breeds were found with lesions, while the frequency among various breeds was as follows: the Cocker Spaniel 13, Pointer 4, Beagle 4, Chow-Chow 3, and mixed Shepherd and Collie 5 (graph 1).

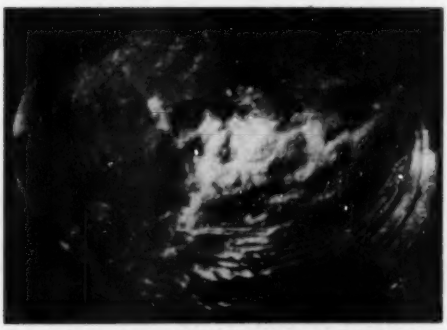


Fig. 2—A cross section of a disc demonstrating a large ventral herniation tract and a smaller dorsal one.

Fig. 3—A cross section of a disc with hypertrophy of the dorsal longitudinal ligament. Some of the dorsal annulus fibrosus is also involved in this protrusion.

A high incidence of disc abnormalities has also been reported in human cadaver surveys. Haley and Perry[6] report that in 99 spinal columns, 63 showed one or more protrusions. Fifty-three cases were in the cervical region, 7 were thoracic protrusions, and 27 were in the lumbar area. Thirty-six per cent of the cases had both cervical and lumbar involvement.

Hansen[7] has recently reported on a series of 100 cases of suspected disc prolapse. The autopsy findings were charted as to location and age, and the highest incidence was at 7 years of age. This worker divided the dogs into a chondrodystrophoid group made up of the Pekingese, Dachshund, and French Bulldog, and into a nonchondrodystrophoid group of dogs including all the other breeds. The

incidence of disc protrusions was markedly higher in the chondrodystrophoid type.

Although no breed is exempt from disc protrusions, more cases are seen among the so-called chondrodystrophoid group except in our case, the Cocker Spaniel and Beagle. The high frequency in Cocker Spaniels can possibly be explained by the great numbers of this breed examined in American clinics.

However, in the clinic where the author of this paper did his research, the percentage of various breeds affected with disc protrusion neuropathies was as follows:

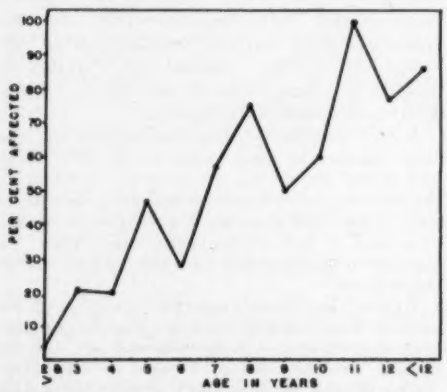
Cocker Spaniel	2.0 %
Dachshund	8.3 %
Pekingese	23.0 %
Beagle	1.8 %

It is felt that the Pekingese figure is erroneous because of the few numbers of this breed seen here and that the cases seen were mostly referred from other veterinarians. Whether the Cocker Spaniel and Beagle would fit into Hansen's chondrodystrophoid group of dogs is questionable since he fails to mention these breeds (graph 3).

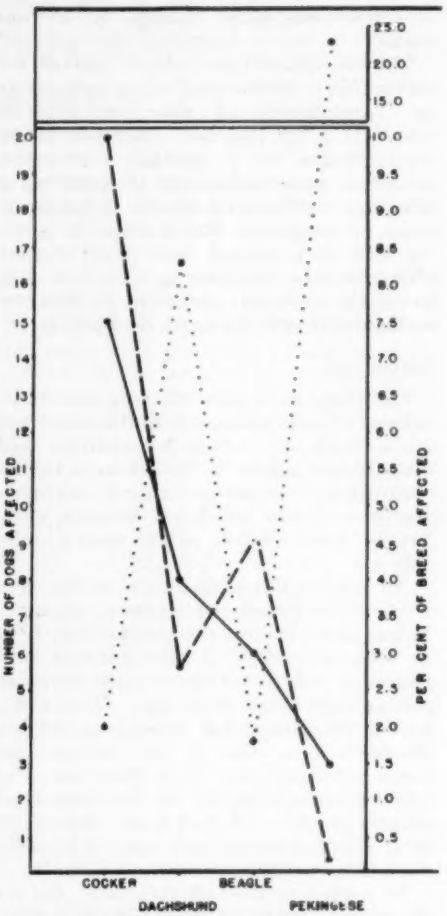
Clinically, this author has studied 46 cases of proved disc protrusion occurring in 14 breeds of dogs, 1 cat, and 1 rabbit. The age incidence of disc protrusion in these clinical cases follows fairly closely that of his autopsy survey, and of Hansen's work.[6] The highest clinical frequency was at 3 years of age (11 out of 46 cases, or 24%) (graph 2).

The incidence among the sexes in the postmortem survey was males, 55 per cent and females, 45 per cent. In 46 clinical cases, the frequency was 24 males, 52 per cent, and 22 females, 48 per cent. Of the 15 Cocker Spaniels affected, however, 12 were females. Although it is claimed that males are more frequently affected, the slight predominance in the male frequency here is not thought to be conclusive.

Protrusions of the intervertebral disc can occur in the cervical, terminal thoracic, and in the lumbar segments of the spinal



GRAPH 1—Age incidence—disc protrusions. Autopsy survey, 130 dogs.



——— No. of dogs per breed affected.
– – – – Per cent of breed seen in total cases.
..... Per cent affected in each breed.

GRAPH 3—Breed incidence—clinical protrusions.

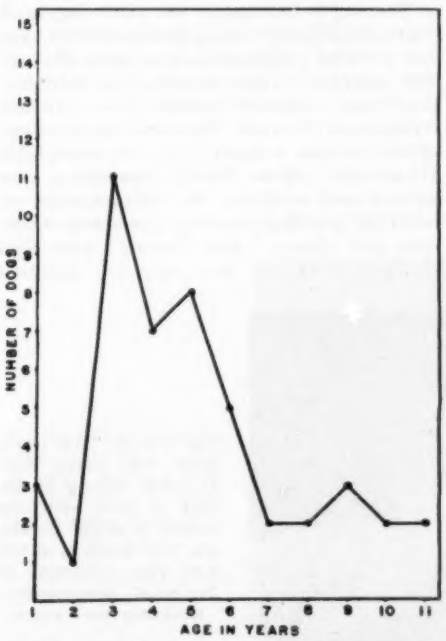
column. The most frequent protrusion site in clinical cases was at the 13T-1L level, while in the autopsy survey the distribution of protrusions was quite uniform throughout the lumbar discs (table 1).

It is felt that one of the major reasons for the absence of disc protrusions in the midthoracic area is because of the presence of the ligamentum conjugale costarum. This ligament is described by Baum and Zeitzschmann [1] as part of the ligamenta capituli costae radiatum. It is a well-developed ligament which extends in a groove across the dorsum of the intervertebral disc under the dorsal longitudinal ligament. This ligament covers the entire width of the disc and largely replaces the annulus fibrosus dorsally. According to Miller,[14] it is absent on the first, eleventh, twelfth, and thirteenth pairs of ribs (fig. 4).

PATHOLOGICAL LESIONS

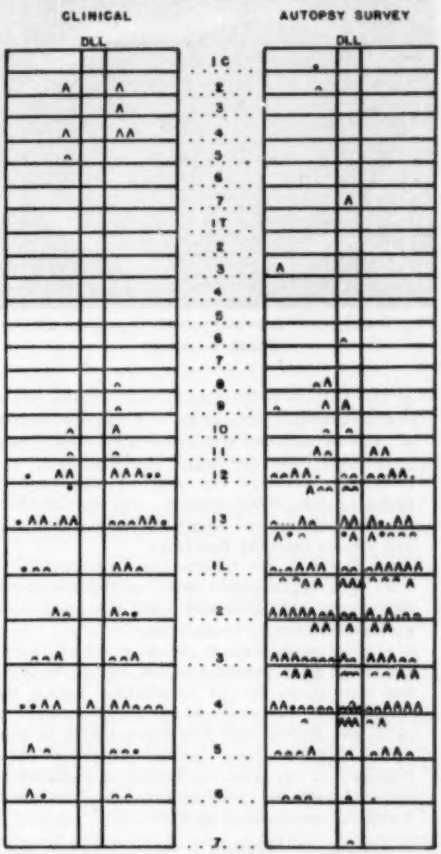
Disc Pathology.—During the course of this research, a comprehensive publication on the pathological-anatomical interpretation of disc degeneration in dogs was published by Hansen. A short summary of this work is as follows:

1) There exists a particularly pronounced predisposition toward this affection in the Dachshund, French Bull dog, and Pekingese. Certain characteristics of chondrodystrophy are found as common denominators of these breeds. He then terms these breeds as the "chondrodystrophied group." The metamorphosis of the nucleus from the gelatinous and hydrous form to the more cartilaginous form present in human old age shows itself to be somewhat different in dogs, with more of a tendency toward chondrodystrophy in some breeds than in other breeds. In this group, there is an early metamorphosis



GRAPH 2—Age incidence—clinical protrusions.

TABLE 1—Distribution of Disc Protrusions



∧—One of multiple lesions over 2 mm. high; ∩—One of multiple lesions 2 mm. high or less; ·—Single lesion over 2 mm.; ˙—Single lesion 2 mm. or less; DLL—Dorsal longitudinal ligament.

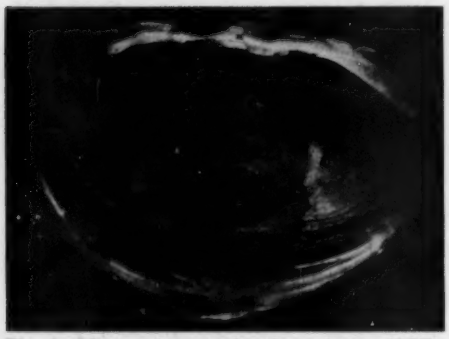


Fig. 4—Ligamentum conjucale costarum (8-9T). Cross section.

of the nucleus to an increase of collagen fibers and a cellular proliferation with the appearance of a nongelatinous chondroid mass resembling youthful hyaline cartilage. This disc at a very early age becomes the seat of degenerative changes, noncellular necrosis, and calcification, since the disc can no longer fill its normal shock and stress-absorbing functions.

2) Disc prolapse follows disc degeneration.

3) Disc degeneration exists in the spinal column much more generally than the number of prolapses allows us to suppose.

4) Disc prolapses, as the most serious manifestations of degeneration, are due to mechanical factors as far as localization within the spinal canal, but multiple localization argues in all other respects that disc degeneration in dogs is a definite systemic disease. To repeat then, tendency of the discs to disease is probably to be explained by predisposition of the above breeds to chondrodystrophy.[6]

Looking at Hansen's work from a clinical point of view, it seems quite logical that there is a breed predisposition to a generalized disc degeneration at a very early age. The author has seen an indication of such degeneration in a 1-year-old Wirehaired Dachshund in which a spinogram showed a slight calcification and narrowing of numerous lumbar discs. The disc which seemed to be prolapsed or protruding evidenced additional narrowing and osteophyte formation of adjacent vertebrae.

From the observations of the author, as well as other workers in America, the Cocker Spaniel and related type spaniels might well belong in this chondrodystrophoid group. And in addition, the condition does exist not too infrequently in all breeds, as Hansen admits; therefore, the syndrome must resemble the human disc degeneration in so-called nonchondrodystrophoid breeds.

This human disc degeneration seems to be a senile change in that there is a close correlation between the elasticity of the nucleus pulposus and its water content. This desiccation seems to be of primary importance in the senile destruction of the disc and the production of pathological lesions.[17,20] This change can be seen grossly in the dog by a loss of the glistening and characteristically wet appearance of a normal nucleus pulposus and annulus fibrosis. The annulus is also involved in dehydration and weakens under the constant stresses and strains. The mucoid structure of the nucleus is replaced by fibrocartilage. Hyndman [9] claims that he has never seen microscopic evidence of inflammation or necrosis in a degenerating disc while Saunders and Inman,[18] and Hansen [7] state that it does occur. At any rate, the annulus

Fig. 5—C38. This spinogram was taken Nov. 16, 1945, during an attack of neck pain and partial brachial paralysis. The lesion is at the 2-3C disc. (Courtesy of Dr. H. C. Stephenson.) Recovery took place.

also degenerates and loses its laminated appearance and structure. It ultimately becomes so thin and inadequate that it can no longer retain the sequestrating nucleus, and the latter is extruded into the spinal canal.

After a prolapse occurs, the question arises as to what happens to the disc. Lindblom and Hultquist [11] state that the disc tissue may be digested and absorbed as it reaches the disc surface in a ruptured annulus, that absorption of the prolapse can occur and therefore symptoms will disappear. This concept then explains the spontaneous recovery seen in an occasional clinical case (fig. 5, 6).

Although absorption of the prolapsed nucleus can be accomplished, calcification can also occur. Disc calcification, however, may not necessarily indicate a disc protrusion but may merely be a result of degeneration and/or necrosis.[15,16] However, a calcified nonprotruding disc may be the source of future rupture of the annulus because of the degeneration which has occurred.

Spinal Cord Pathology.—Chronic Disc Protrusion Paraplegia.—The gross pathology of the spinal cord may not be great. Hemorrhage may be quite profuse extradurally or may be slight. There may be evidence of local subdural or subarachnoid hemorrhage in the protrusion area. The dura frequently forms an adhesion to the protrusion. Calcified deposits on the dura may be present. These adhesions may be the cause of the subarachnoid block which can be demonstrated by contrast myelography. Nerve roots may be embedded in the extradural fibrotic calcified mass if the protrusion extends laterally. In 2 dogs, extreme dural ossification (postmortem survey) was in existence at the pathological lesion site and adjacent to it.

Depending on the duration of the injury prior to autopsy, an extradural necrotic deposit will be seen to some extent. In those cases autopsied shortly after injury (within a few days), this deposit will be one of some extent. It will be a slate gray to a red color, gritty, and shows signs of calcification. From three to four weeks after injury the material may have been completely absorbed or nearly so in that only a few tiny flecks remain on the spinal canal floor. However, the extradural deposit may remain as a calcified mass on the dura or form a bony plate on the floor of the canal.

This extradural deposit microscopically contains erythrocytes, leukocytes, fibroblasts, calcium deposits, fat cells, cartilage cells, and an occasional osteocyte. Much nondescript necrotic material is present. The histopathological examination would suggest an inflammatory necrotic reaction as the result of an irritant and/or trauma (fig. 7).

An effort was made to experimentally determine whether the extradural reaction was due to the expulsion of the nucleus pulposus into the epidural fat. Nuclear material obtained by disc curettage was placed extradurally by means of a trephine

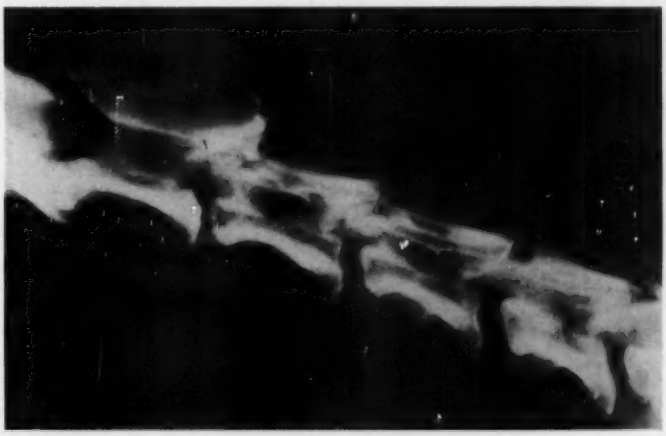


Fig. 6—C38. This spinogram was taken June 26, 1951. Notice the reabsorption of some of the calcified material at 2-3C disc.

opening into the spinal canal. The dogs were then destroyed in two to four weeks. The most typical reproduction of the extradural deposit as seen in clinical cases was obtained when the nuclear material from an aged Cocker Spaniel was placed into the extradural space of a puppy. The microscopic structure of the deposit was indistinguishable to that of a clinical deposit. Then it would seem that trauma is not absolutely necessary for this reaction to take place but that the nuclear substance, especially that which has undergone degeneration, will initiate this extradural reaction (fig. 7).

The cross section of the affected cord over the protrusion may show a gross indentation, hematomyelia, softening, and a grey-red appearance. Microscopically, the white matter appears moth-eaten or sievelike. These areas may show some compound granular corpuscles which are commonly known as "gitter" cells (fig. 8). These cells are swollen microglia or Hortega cells which because of injury to the myelin sheath have assumed the scavenger function of taking up and digesting remnants of broken down nervous tissue. This reaction according to Weil [21] may start within twelve hours after injury and is in "full swing" in thirty-six hours.

Other pathological features which may be present in the cord from disc paraplegia are gliosis around these damaged areas, or some hypertrophy and hyalinization of blood vessel walls. In most cases, this whole picture is quite local and may extend no more than one vertebra to either side of the lesion. Occasionally, isolated demyelination throughout the spinal cord will occur from an ascending or descending demyelination. Neurons may or may not show degenerative changes.

The above description is based on the pathology observed by the author in the spinal cord segments associated with pro-

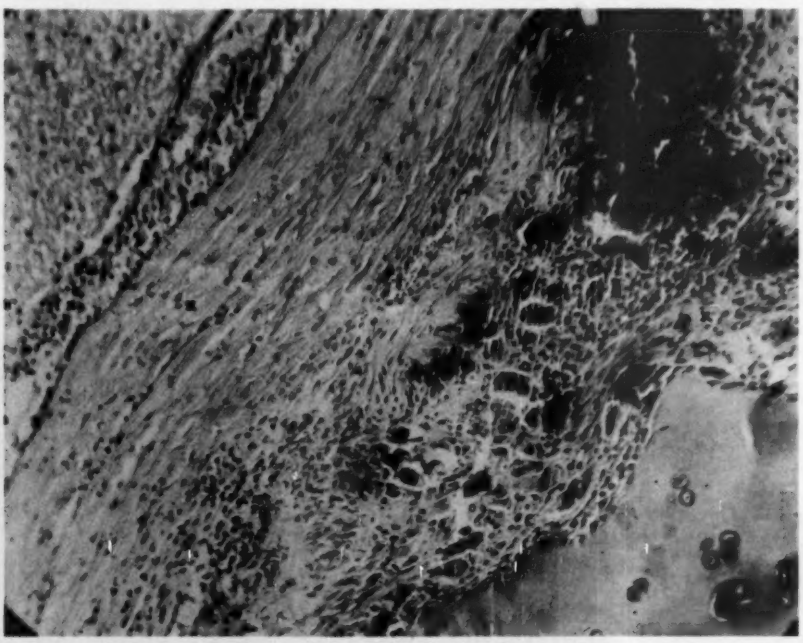


Fig. 7—A microscopic section of an extradural deposit resulting from placing nucleus pulposus upon the dura mater (artificial deposit). This is, however, typical of a natural deposit from disc protrusion. From extreme left to right is cord tissue; a characteristic leptomeningitis from the use of pantopaque in the subarachnoid space; dura mater; and the extradural deposit demonstrating calcification necrosis, fibrosis, and cartilage. (High dry).

truding discs and corresponds to descriptions by human texts for compression demyelination. Fankhauser [4,5] mentions some of these pathological changes. McGrath [12] calls this syndrome a "focal epidural pachymeningitis with cord contusion." However, veterinary literature does not offer a complete description of the gross and microscopic pathology of the spinal cord in disc protrusion paraplegia.

As to the cause of this pathology, most authors agree that circulatory disturbances are responsible for breaking down of the white matter. Compression of blood and lymph vessels is seen in some areas of disc injury. Therefore, local anemia and stasis of blood and lymph fluid result. This produces the edema and softening of the sensitive myelin sheaths, with a subsequent demyelination.[21]

Acute Disc Protrusion Causing a Progressive Paralysis.—This condition is one of great interest and shows a marked pathological as well as clinical picture. According to Innes,[10] the maximum functional disturbance of any nervous condition occurs when the course is of an extremely rapid development.

This condition has been mentioned in veterinary literature by various workers, among whom are Fankhauser,[4,5] McGrath,[12] and Hutyra *et al.*[8] However, since these descriptions have been brief and inadequate pathologically, as well as clinically, considerable time and space will be devoted here to its description.

This condition *per se*, that is as a result of disc protrusion, does not exist in man. Of the 5 clinical cases observed by the author, the pathological picture has been typical. Grossly, there is a diffuse reddish gray extradural necrosis and a subdural hemorrhage usually existing throughout entire length of the cord. A ruptured disc on the floor of the canal can be demonstrated as a rent or tear in the dorsal annulus with little or no local protruding mass. Extradural hemorrhage may be marked, especially around the disc rupture.

A hemotoxylin and eosin-stained section shows a diffuse softening, hemorrhagic infiltration, extreme demyelination, and large cavitations in the cord (fig. 9). Degenerated neurons and a marked polymorphonuclear infiltration, especially neutrophilic, in the cord substance can be seen. The picture is largely one of severe concussion; however, in no instance was there the slightest indication of accident or violent trauma as related by the owner. Bacterial cultures were run on the extradural deposits and on the cord substance of 1 dog, and no organisms were found except some gram-positive anaërobic rods. The

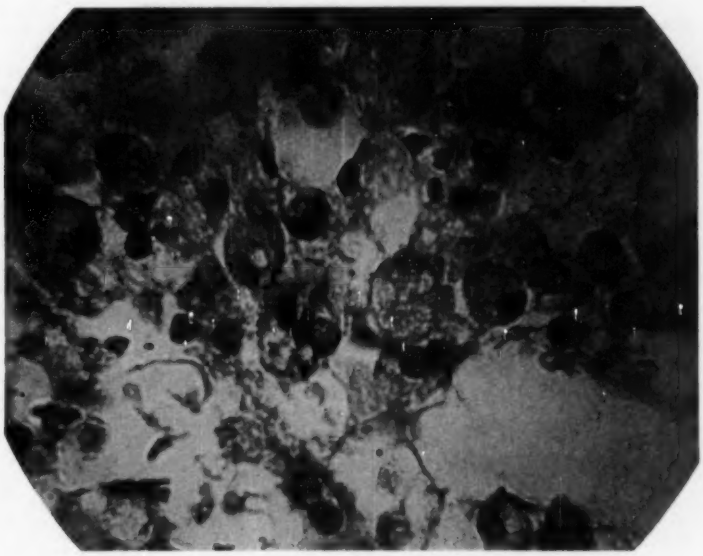


Fig. 8—C1. "Gitter cells" present in demyelinated nerve tissue, the result of a disc protrusion. (High dry).

presence of these organisms can possibly be explained by agonal invasion, since this dog was in a comatose and paralytic state for two days before death. In 3 of the other cases, there were no bacteria seen on hemotoxylin and eosin-stained sections or on tissue bacterial stains of these sections. Therefore, it would seem that the inflammatory polymorphonuclear infiltration was a response to tissue necrosis or chemical change, or at least not due to an overwhelming bacterial invasion.

The pathological changes are difficult to explain by the presence of a ruptured disc. However, there is no evidence of outside trauma or concussion to explain the cause of the changes. Neuropathologists attribute such changes as due to a generalized "shaking-up" of the nervous elements. In man, hemorrhages may occur by diapedesis or from a preëxistent vascular change. Weil [21] proposes that a sudden extension and injury to the vasomotor nerves of the vessels entering the spine might cause a general vasodilation with stasis. This would result in ischemia to the cord and resultant softening. However, the hemorrhage in the condition existing in the dog seems to be, at least, a local rupture of vessels with frank hemorrhage.

Then it might be stated that the acute disc rupture and subsequent progressive paralysis produces a fulminating cord reaction corresponding to that of a concussion. Thus far, the pathological changes can be explained by a ruptured intervertebral disc.

SUMMARY

1) Dorsal protrusions of the intervertebral disc can be seen as herniations of the nucleus pulposus with or without rupture of the annulus, edema of the disc, hypertrophy of the dorsal longitudinal ligament from herniation of the disc under it, or associated with a dorsal spondylitis deformans.

2) The incidence of clinical disc protrusion in the dog was 0.7 per cent. Each sex was about equally affected. The so-called chondrodystrophoid group of dogs was most frequently affected. Dogs from 2 to 10 years of age were affected while the most frequent age observed clinically in 46 cases was 3 years. However, in the postmortem survey of 130 normal dogs of all ages, the incidence of abnormal discs was 41.5 per cent, of which 87 per cent of the positive dogs were 5 years of age or older. Therefore, a relatively small percentage of dogs with pathologically abnormal discs showed clinical symptoms during the time that these observations were made.

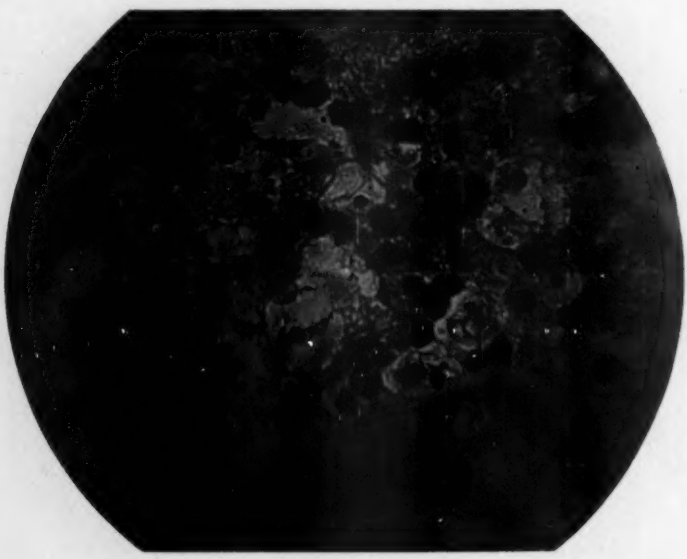


Fig. 9—C7. An acute disc protrusion paralysis which demonstrated neutrophilic infiltration of the white substance of the cord (oil).

3) The disc frequently affected was the 13T-1L; however, protrusions may also occur in the cervical as well as lumbar segments. The midthoracic segment is not usually affected and this phenomenon is probably largely explained by the presence of the ligamentum conjugale costorum in this region.

4) In Hansen's so-called chondrodystrophoid group of dogs (Dachshund, Pekingese, French Bulldog), a systemic metaplasia of the disc from a hydrous mucoid to a cartilaginous form predisposes the exciting injury for disc protrusion. In other breeds, a condition of senile dehydration and disc degeneration precedes the exciting cause (as in man).

5) Characteristic epidural necrotic deposits, as seen clinically in rupture of intervertebral discs, were reproduced experimentally by placing the nucleus pulposus substance on the dura mater. The degenerated nucleus pulposus of an aged Cocker Spaniel produced a deposit indistinguishable from that of clinical lesions.

6) The spinal cord pathology resulting from the chronic disc protrusion paraplegia as well as the acute disc protrusion causing a progressive fatal paralysis is described.

References

[1] Baum, H., and Zeitzschmann, O.: Anatomie des Hundes, 1 Band, Skelett und Muskelsystem. Paul Parey, Berlin, (1936): 21.

[2] Deucher, W. G., and Love, J. G.: Pathologic Aspects of Posterior Protrusions of the Intervertebral Discs. Arch. Path., *27*, (1939): 201-211.

[3] Donohue, W. L.: Pathology of the Intervertebral Disc. Am. J. M. Sci., *198*, (1939): 419-436.

[4] Fankhauser, R.: Zur Nachhandlähmung beim Hunde. Schweizer Arch. f. Tierheilk., *90*, (1948): 494.

[5] Fankhauser, R.: Uber die Sogen. Dackellähmung. Schweizer Arch. f. Tierheilk., *90*, (1948): 143.

[6] Haley, J. C., and Perry, J. H.: Protrusions of Intervertebral Discs, a Study of Their Distribution, Characteristics and Effects on the Nervous System. Am. J. of Surg., *80*, (1950): 394-404.

[7] Hansen, Hans-Jörgen: A Pathologic-Anatomical Interpretation of Disc Degeneration in Dogs. Acta. orthop. Scandinav., *20*, (1951): 280.

[8] Hutyra, F., Marek, J., and Manniger, R.: Special Pathology and Therapeutics of the Diseases of Domestic Animals. 5th ed. Alexander Eger, Inc., Chicago, (1946): 362-388.

[9] Hyndman, O. R.: Pathologic Intervertebral Disk and its Consequences. Arch. Surg., *53*, (1946): 247-297.

[10] Innes, J. R. M.: Neurophysiology and its Clinical Application to Nervous Disorders of Animals. North Am. Vet., *33*, (1952): 171.

[11] Lindblom, K., and Hultquist, G.: Absorption of Protruded Disc Tissue. J. of Bone and Joint Surg., *32-A*, (1950): 557-560.

[12] McGrath, J. T.: Spinal Paralysis of the Dog. North Am. Vet., *32*, (1951): 700.

[13] Miller, M. E.: Guide to the Dissection of the Dog. 2nd ed. Edwards Bros., Inc., Ann Arbor, Mich., *14*, (1948): 367.

[14] Miller, M. E.: Personal communication.

[15] Olsson, Sten-Erik.: On Disc Protrusion in Dog (Enchondrosis Intervertebralis). Acta orthop. Scandinav. Supplementum No. 8, Ejnar Munksgoard, Copenhagen, 1951.

[16] Pommer, A.: Die Spondylitis deformans und Spondylarthritis ankylopoetica bei Hunden und Katzen in Rontgenbilde. Wien. tierärztl. Monatschr., *5*, (1933): 129.

[17] Püschel, J.: Der Wassergehalt normaler und degenerierter Zwischenwirbelscheiben. Beitr. z. path. Anat. u.z. allg. Path., *84*, (1930): 123-130.

[18] Saunders, J. B., and Inman, V. T.: Pathology of the Intervertebral Disc. Arch. Surg., *40*, (1940): 389-416.

[19] Schmorl, G.: Zur pathologischen Anatomie der Wirbelsaule. Klin. Wchnschr., *8*, (1929): 1243-1249.

[20] Uebermuth, H.: Die Bedeutung der Altersveranderungen der menschlichen Bandscheiben für die Pathologie der Wirbelsaule. Arch. f. klin. Chir., *156*, (1929): 567-577.

[21] Weil, A.: Textbook of Neuropathology. 2nd ed. Grune & Stratton, New York, (1945): 214-218, 112, 36.

Intervertebral Disc Protrusions in the Dog
II. Symptomatology and Clinical Diagnosis

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FROM PART I, "Incidence and Pathological Lesions" of the "Intervertebral Disc Protrusions in the Dog,"[6] it was seen that disc protrusions are a frequent cause of spinal cord injuries. Also, the pathological responses to these injuries as well as abnormal predispositions of the disc to injury were explained. It is the purpose of this paper to concisely relate those symptoms seen clinically in various disc protrusion conditions and the means of making a purely clinical diagnosis.

CLINICAL SYMPTOMATOLOGY

The clinical symptomatology exhibited by a dog suffering from disc protrusion can vary considerably.[3, 13] When the question is placed before the owner as to how this occurred, the most frequent answer is to the effect that the dog was fine yesterday but was found in this condition this morning. Occasionally, a sudden unusual movement will precipitate such injury, such as jumping upon the bed, jumping over a fence, going upstairs, tussling with the neighboring dog, or breeding. Love and Walsh[10] found that 84 per cent of 500 human cases had intermittent symptoms and remissions. Similarly, the dog will often recover spontaneously only to suffer one or more relapses in the future.

The categories of symptom complexes may be referred to as (1) pain; (2) paresis and/or incoördination; (3) paraplegia, spastic or flaccid; (4) progressive ascending paralysis from an acute disc prolapse.

Under the pain syndrome, the dog may exhibit abdominal pain, lumbar or localized pain, or generalized pain. Kyphosis or arching of the back may occur in an effort to decrease the dorsal projection of the disc. Rarely a scoliosis or lateral spinal curvature with the convexity occurring away from the lesion side is seen.

When in pain, the dog will object to being forcibly moved or picked up, to walking upstairs, or jumping into a car. He may cry and awaken frequently throughout the night. Reflexes in this stage are usually exaggerated and the limb as well as the dorsal musculature may be spastic.

Pain may also be present in the state known as paresis. Paresis is defined by Dorland[2] as a weakness or incomplete paralysis. This condition may be manifested by posterior incoördination if the lesion is bilateral or by the dragging of a toe or leg in extension if the lesion is unilateral. In an effort to compensate for a unilateral paresis, the lumbar spine may show a scoliosis with the convexity being away from the affected part. In cervical disc protrusion paresis, all limbs may show an affectation. Occasionally, along with cervical protrusions, a unilateral brachial paresis or paralysis may occur in addition to neck pain or a general hyperesthesia.

A condition known as paraplegia (hind leg or posterior extremity paralysis) may result separately or concomitantly with pain. If the musculature of the posterior limbs are spastic, as manifested by exaggerated reflexes and sensation, the condition is termed as spastic paraplegia. Spastic paraplegia is usually associated with pain. Here the dog will scoot or carry his hind legs under him since he is not willing to flex them. Urine and fecal retention usually occurs in this condition. This retention may be due to the dog's inability to get into the usual posture for these acts so as to make use of the necessary skeletal musculature, or it may be due to a direct disturbance of the micturation and defecation spinal centers.

A flaccid paraplegia, or posterior paralysis, is one in which the musculature lacks tonus, reflexes and sensations are diminished, and the urination and defecation sphincters show a lack of tonus which results in an incontinence or dribbling of urine and feces. Here the legs are carried behind the body instead of under the body as in spastic paraplegia. Severe muscle atrophy takes place, decubital ulcers occur, and prognosis in general is unfavorable.

This paper is an excerpt from a thesis presented to the faculty of the Graduate School of Cornell University for the degree of doctor of philosophy in June, 1952. This work was supported by the Research Council of the American Veterinary Medical Association.

The final category of symptomatology might be called the acute progressive paralysis initiated from an acute severe disc rupture. Here, the onset is sudden with rapid development of sensory and motor paralysis of the tail, croup, and hind legs, and incontinence of urine and feces. Progressive paralysis extends to the thorax where respiration becomes diaphragmatic. Forelegs become spastic and then flaccid, and finally death ensues undoubtedly from respiratory failure either from diaphragmatic or bulbar paralysis. The prognosis, of course, is unfavorable.

In general, the symptomatology will depend on the location of the lesion and the degree of pathological response to that disc protrusion. The term "pathological response" is used here instead of "extent of injury," since no 2 dogs will react similarly to a given protrusion. This individual variation as to the response and adaptation or compensation of the nerve tissue involved in the compression makes accurate prognosis difficult.

The usual progression in symptoms in a typical subacute or chronic disc injury with intervening improvements and recurrences is pain, altered coördination and function, spastic paraplegia, and finally flaccid paraplegia. Aside from these symptoms, the dog may exhibit little other constitutional dysfunction but can be relatively normal.

Neurological Signs

The use of clinical diagnostic methods in the dog is limited. In man, the "slipped disc" syndrome is usually a matter of pain, or pressure on a nerve root, and is called "sciatica." However, in the dog, the condition is characterized also by paresis and/or paraplegia, since the pressure usually is directly upon the spinal cord. According to Brock,[1] if these symptoms were obtained in man, a neoplasm, or some lesion other than a herniated disc, would be suspected.

If the general clinical picture suggests a spinal cord compression, a neurological examination is helpful. In human sciatica, 80 per cent of these cases can be diagnosed by neurological examination alone. In about 90 to 95 per cent of the above group, the lesion can be accurately localized clinically to the fourth lumbar or lumbosacral level. Then perhaps in 30 per cent of these can exact localization be made. There seems to be some controversy among human neurosurgeons as to whether the result of such findings should be confirmed by contrast spinograms.[1] At any rate, the art and science of securing the sensation and reflex findings in man is highly developed.

However, in the dog such neurological findings are more difficult to evaluate. The dog is not so coöperative in obtaining a true relaxed response. The animal is in strange surroundings, frightened, and is either showing abnormal responses or is exhibiting cortical inhibition of reflex responses. If, however, the animal is in quiet surroundings and the veterinarian has gained the animal's confidence, helpful information can be elicited by reflex and sensation responses. However, the author concurs with the opinion of Frauchiger and Fankhauser[4] that localization of the injury is most difficult by neurological means.

The reflexes employed routinely for paraplegia are as follows.[5]

1) Patellar.—With the animal on his side, the upper leg is supported lightly on the medial side of the femur or stifle, a sharp light blow is placed on the crural tendon between the patella and tibial tuberosity. The lower limb is brought forward.

2) Supratarsal.—The dog is placed in the same position; the tibialis anterior and the long digital extensor tendon is struck proximal to the hock. The hock is flexed and the toe is extended.

3) Achilles.—The dog is in same position and the Achilles tendon is struck proximal to the tuber calcis. The hock is extended.

4) Pedal Flexion Reflex.—The dog is in the same position; the skin between the toes is pinched and the leg is retracted sharply. A variation of this is the plantar reflex in which the skin of the sole between the pads is tickled. The toes flex.

5) Scratch Reflex.—Scratch the lateral thoracic or abdominal wall of the recumbent animal and the hind limb will be alternately flexed and extended.

Other reflexes used occasionally were the extensor thrust, crossed extensor reflex, and all the corresponding foreleg reflexes when a cervical protrusion was suspected.

While the reflexes used above were of value in diagnosis, the superficial responses to needle pricking were of far more value in localization.[3] Here the dog was observed for exaggerated twitching (hyperesthesia) or lessened twitching (hypesthesia). Paresthesia (morbid or depraved sensation) is not as easily observed as in man since the dog can not describe or tell about these variations. One can readily see that a nor-

mal dog has rather good skin sensation in the thoracic region but has lessened sensation in the lumbar and hind leg areas. There is a wide variation of responses in individuals. If, however, a small area is distinctly hyperesthetic, this is an indication of nerve pain and injury. Typically, hyperesthesia occurs anterior and hypesthesia posterior to pathological lesion. The lesion in some cases may be one to two vertebrae ahead of the sensation change because of the nerve root progression in the canal before its emergence. In other instances, the sensation change is quite accurately over the lesion. In any case, it can be seen that an approximate area can be determined neurologically (table 1).

DIFFERENTIAL CLINICAL DIAGNOSIS

Although clinical forms of disc protrusions are usually quite easily diagnosed from the history, breed occurrence, and symptoms shown, other conditions and diseases which may confuse the diagnosis are listed as follows:

1) Inflammatory infectious diseases involving the spinal cord.
   (a) Rabies, with a progressive paralysis in later stages.
   (b) Myelitis and meningitis.
      1) Canine distemper.
      2) Secondary infections, metastatic.
2) Noninfectious inflammatory conditions.
   (a) Abscesses.
   (b) Dural ossification (pachymeningitis ossificans). If this condition occurs with no relation to disc protrusion, it usually attacks the larger working breeds. It is an insidious, progressive disease. Bony plaques causing pressure on the cord and the nerve roots produce the symptoms shown.[8]
   (c) Spondylitis deformans ossificans. These conditions may cause pressure to the nerves leaving the spinal canal or cause pressure on the cord itself by dorsal exostosis. Spondylitis is common in old and large breeds of dogs especially and can be differentiated by spinograms.[8, 12]
3) Degenerative conditions of the cord.
   (a) Avitaminosis.
      1) Avitaminosis B. Deficiency of niacin, thiamin, and riboflavin have all been demonstrated to cause degenerative lesions in the central neurons and to cause impaired nerve response.
   (b) Demyelination conditions.
      1) "Old dog" encephalitis. A demyelination of brain and spinal cord in adult and old dogs.[7, 11]
      2) Postdistemper demyelination. This is similar to the above description but has a history of following distemper.
      3) Postvaccination (rabies) paralysis. This is weakness to a complete paralysis following the use of phenolized rabies vaccine.[7]

TABLE 1—Reflex Responses from Affected Discs—Clinical Cases

REFLEX	TOTAL CASES GIVING ABNORMAL REFLEXES	NUMBER HYPER-ESTHETIC	NUMBER HYPO-ESTHETIC	PERCENTAGE OF ABNORMAL REFLEXES PER AFFECTED DISCS								
				3-4C (1)*	4-5C (1)	10-11T (2)	12-13T (5)	13-14L (6)	2-3L (2)	3-4L (3)	4-5L (4)	5-6L (2)	6-7L (1)
PATELLAR	19	11	8			100	80	84	100	66	50	50	100
ACHILLES	14	8	6			50	80	50	50	0	75	50	100
TARSAL	17	12	5			100	80	66	50	66	50	50	100
PEDAL	21	12	9			100	80	84	100	66	100	50	100
SCRATCH	21	0	21			100	80	100	50	66	100	50	100
URINE	17	2 INCONTINENCE	15 RETAINED	100		100	80	50	50	33	75	50	100
FECES	14	6 INCONTINENCE	8 RETAINED	100		100	80	16	50	33	75	0	100
TAIL	10	0	10			100	60	16	0	0	50	50	100
PAIN WITHIN 1 VERTEBRA OF LESION	15	14	1	100	100	0	66	40	100	66	50	50	0

* NUMBER OF CASES

4) Traumatic injuries of the cord and spinal column.
   (a) Fractures.
      1) Compression.
      2) Transverse.
   (b) Luxations.
5) Neoplasms.
   (a) Extradural.
   (b) Cord neoplasms.
6) Congenital conditions of the cord and spinal column.
7) Nonspecific.
   (a) Toxemias.
      1) Constipation, obstipation.
      2) Unknown causes. These cases usually are more in the nature of a general paresis rather than a posterior paresis or paraplegia.

In regard to the above conditions listed as possibilities concerned in establishing a diagnosis, the question arises as to what proportion of dogs showing paresis, pain, and paraplegia are due to protruding discs. The diagnostic records of the small animal clinic at the New York State Veterinary College were examined for an eighteen-month period extending from July 1, 1950, to Jan. 1, 1952. During this period, the author was conducting his research and was consulted or conducted the diagnostic routines on many of these cases. However, in many of the case histories in the files, it was difficult to properly evaluate the cause of the condition. Positive causes were often difficult to establish under the circumstances. The approximate figures are as follows:

Total Cases 8,233

1) Intervertebral disc protrusions	41	(52 %)
2) Other conditions showing similar symptoms	38	(48 %)
(a) Luxation	2	
(b) Myositis	4	
(c) Undeterm, posterior paresis	13	(1 cat)
(d) Spondylitis	1	
(e) Fractures	2	
(f) Taeniasis	2	
(g) Postdistemper paralysis	1	
(h) Medulloblastoma	2	
(i) Toxemia	1	
(j) Constipation	3	
(k) Undeterm, general paresis	4	
(l) Spinal cord concussion	1	
(m) "Old dog" encephalitis	2	
Total	38	

The above cases were all symptomatically confused with protruding disc paraplegia. These cases represent all ages and all breeds. The disc paraplegia cases (41) were all proved by x-ray and/or autopsy. It will be noted that approximately 50 per cent of this clinical group of conditions are due to disc protrusions.

Clinical Pathology

In an attempt to explore all the possible aids in the diagnosis of disc protrusion paraplegia, clinical diagnostic tests were employed in the clinical cases cited. Since the condition involves the central nervous system, analysis of spinal fluid would seem logical.

The normal spinal fluid characteristics in the dog were determined as:

Spinal pressure (under anesthesia)—97 mm. Ringer's solution.
Specific gravity—1.0054.
w.b.c.—3 per cubic millimeter.
Pandy test—negative to a very slight reaction.
Appearance—clear, no clot.

Other normal determinations according to Frauchiger and Fankhauser are:[4]

Total protein (quantitative)—30 mg./100 cc.
Sugar—50 to 75 mg./100 cc.
Chlorides—675 to 750 mg./100 cc.

In brief, positive diagnostic aids in spinal fluid analysis are not usually present in disc protrusions. A rise of spinal fluid pressure and a positive Pandy test may occur. However, these tests listed above are of value in ruling out other conditions which may be confused with disc protrusions, such as in meningeal conditions where an altered w.b.c. and protein concentration can be seen.

In blood and urine analysis, no pathognomonic results are gained. It has been stated that an elevated blood nonprotein nitrogen level is indicative of disc protrusion. However, in only one-third of the cases could a reading of over 20 mg./100 cc. of blood be obtained. These could be explained by a previous nephritis, an acute paralysis with hemoconcentration, or a chronic urine retention. It is believed that primary nephritis has little to do with disc protrusions.

An interesting but unexplained blood Ca/P relation was seen in some cases. Of 10 dogs where serum P levels were run, 7 showed readings of above 4 mg./100 cc. of blood while 3 of these dogs had readings above 11 mg. per 100 cc. of blood. The corresponding Ca levels were relatively normal. From the literature, possibly urine

retention and cystitis might explain this relationship.[9]

In conclusion, one might say that blood, urine, and spinal fluid analyses show little significant change in the disc protrusion. These determinations are, however, an aid in differentiating disc protrusions from related spinal conditions.

DISCUSSION OF CLINICAL CASE HISTORIES (45 DOGS, 1 CAT)

It will be seen from the clinical case histories that 9 dogs (20 %) had suffered previous occurrences of illness from disc protrusions and had at least partially recovered, only to have a recurrence. Thirty-six dogs (80 %) had no such previous diagnosis. It can be concluded that most dogs have no history of injury associated with the initial attack.

Of 25 dogs affected with a paraplegia or general paresis, 23 dogs (92 %) suffered from urine and fecal retention or incontinence. Of those cases showing pain or partial paresis, 1 out of 20 (5 %) suffered from excreta incontinence. One dog seemed to suffer from urine incontinence exclusively as a result of lateral spondylitis exostosis at 5-6L disc. It was relieved by surgury. No other therapy was employed.

The reflex responses obtained from these cases were analyzed and the abnormal responses were tabulated (table 1). It will be seen that most cases showed some impairment of reflexes; for instance, 46 per cent showed abnormal pedal reflex while 33 per cent showed abnormal cutaneous sensation within one vertebra of the primary lesion. In the former case, the reflex abnormalities demonstrated that some abnormal nervous lesion was present but told little concerning the location, since the responses were seen on widely scattered locations. However, the skin reflex is an aid in determining the lesion location in at least one-third of the cases. To further emphasize the clinical reflex diagnostic weaknesses, some clinically normal dogs will show some abnormal reflex and cutaneous sensation responses.

In the differential diagnosis of these clinical cases, all but 1 disc protrusion case was diagnosed correctly before autopsy. However, 1 case was diagnosed as disc protrusion paraplegia clinically and on x-ray but pathologically was medulloblastoma.

SUMMARY

1) The clinical symptoms seen in the following forms of disc protrusion neuropathies are discussed: (a) pain, (b) paresis and/or incoördination, (c) paraplegia, spastic or flaccid, (d) progressive ascending paralysis from an acute disc rupture.

2) Neurological reflexes and cutaneous sensations were discussed. It was seen that the cutaneous pain sensations are helpful in locating the lesion while the other reflexes aid in making a pathological evaluation and a prognosis of a particular case.

3) Blood, spinal fluid, and urine diagnostic tests have no significant positive results in establishing a diagnosis but aid in differentiating this condition from related ones.

4) From clinical case history summaries (45 dogs, 1 cat) it was seen that 20 per cent of the cases have had previous attacks, that 92 per cent of the paraplegics have urine and fecal retention or incontinence, and that about 50 per cent of all cases showing spinal symptoms result from disc protrusions.

References

[1] Brock, S.: Injuries of the Brain and Spinal Cord and their Coverings. 3 d ed. Williams & Wilkins Co., Baltimore, Md., (1949): 584, 601.

[2] Dorland, W. A. N.: The American Illustrated Medical Dictionary. 18th ed. W. B. Saunders & Co., London, Philadelphia, Pa., 1940.

[3] Fankhauser, R.: Zur Nachhandlähmung beim Hunde. Schweiz. Arch. f. Tierheilk., *90*, (Sept., 1948): 494.

[4] Frauchiger, E. and Fankhauser, R.: Die Nervenkrankheiten unserer Hunde. Schmid & Cie. Bern., (1949): 49.

[5] Habel, R. E.: Diagnostic Reflexes. Applied Anatomy (Class Notes), (1950): 29.

[6] Hoerlein, B. F.: Intervertebral Disc Protrusions of the Dog. Part I. Incidence and Pathological Lesions. Am. J. Vet. Res., *14*, (1953): 260-269.

[7] Jervis, G. A., Burkhart, R. L., and Koprowski, H.: Demyelination Encephalomyelitis in the Dog Associated with Antirabies Vaccination. Am. J. Hyg., *50*, (1949): 14.

[8] King, L. S.: Disseminated Encephalomyelitis of the Dog. Arch. Path., *28*, (1938): 151.

[9] Kolmer, J. A.: Clinical Diagnosis by Laboratory Examinations. 2nd ed. Appleton-Century-Crofts, New York, (1949): 131.

[10] Love, J. G. and Walsh, M. N.: Intraspinal Protrusions of Intervertebral Disks. Arch. Surg., *40*, (1940): 454-484.

[11] Olafson, P.: Class Notes in Special Pathology. New York State Veterinary College, Cornell University, Ithaca.

[12] Pommer, A.: Die Spondylitis deformans und Spondylarthritis ankylopoetica bei Hunden und Katzen in Rontgenbilde. Wien. tierärztl. Monatschr., *5*, (Marz 1933): 129.

[13] Riser, W. H.: Posterior Paralysis Associated with Intervertebral Disc Protrusion in the Dog. North Am. Vet., *27*, (1946): 633-642.

Intervertebral Disc Protrusions in the Dog
III. Radiological Diagnosis

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ALTHOUGH IT has been seen in previous articles[9] that intervertebral disc protrusions have a rather typical history and a characteristic pattern as to age, breed, and symptomatology, the diagnosis of the lesion site and the more revealing facts as to diagnosis and prognosis must be made by x-ray photography.

Roentgenological examinations greatly facilitate the diagnosis of intervertebral disc protrusion disorders. These findings, however, should be correlated with the neurological examinations for more accurate evaluation, since multiple lesions are frequently found. Some cases, especially those of recent injury, can not be properly diagnosed except by the use of contrast medium.

NORMAL CONTRAST

Normal contrast spinograms should be taken from at least two positions. The usual positions are the lateral and dorsoventral (dog on his sternum) while oblique x-ray pictures of the thoracic vertebrae may be helpful. While a lateral view gives the best view of the disc spaces, the dorsoventral view differentiates pathology in the disc from that outside the disc or spinal canal. Oblique views of the thoracic area make the disc more visible since the ribs do not obstruct vision.

This paper is an excerpt from a thesis presented to the faculty of the Graduate School of Cornell University for the degree of doctor of philosophy in June, 1952. This work was supported by the Research Council of the American Veterinary Medical Association.

The utmost care should be used in roentgenological examinations, both in the technique used and in the interpretation. The equipment need not be elaborate, since a good share of the author's investigation was done on a 10 MA, 50 kv.p. portable machine. However, it is the author's opinion that a stationary grid or a Potter-Bucky diaphragm is most helpful in decreasing scattered radiation effects obtained especially in larger dogs.

The roentgenographic changes concerned in spinograms may be listed as:

1) Calcification of the disc, calcification of disc protrusion, or calcification in the intervertebral foremen (fig. 1).

2) Narrowing of the intervertebral space (fig. 2).

3) Osteophytes of the adjacent vertebrae (exostosis on the dorsal vertebral borders).

4) Ossifying deformitive spondylitis (lateral and/or ventral) (fig. 2).

Extensive work on these changes in the dog has been done by Olsson.[12] This investigator has tabulated findings of roentgenograms of 33 dogs involving 54 discs. For the sake of comparison of results between the work of Olsson and the author of this paper, the data will be tabulated side by side on the following page.

Of these 23 dogs upon which autopsies were performed, 49 protrusions or 2.1 per dog were seen. Eighteen out of 49 (37 %) were abnormal on normal contrast spinograms and the remainder, 31 (63 %), showed no protrusion. Three discs were

Fig. 1—C27. Thorotrast given in the cisterna magna shows a marked protrusion at the 3-4L, and a slight protrusion at the 4-5L. These findings were verified completely on autopsy.

TABLE 1— Spinogram Results

Interpretation	Olsson		Hoerlein			
	33 dogs, 54 discs		Autopsy confirmation (23 dogs, 64 discs)		15 dogs,* 35 discs	
	Affected (No.)	Affected (%)	Affected (No.)	Affected (%)	Affected (No.)	Affected (%)
No change	12	22.2	24	38.0	9	26
Diminished IV space	8	14.8	4	6.2	1	3
Dim. space + Ca disc	2	4.0	3	4.6	1	3
Dim. sp. + Ca protrusion	1	2.0	0	0.0	0	0
Dim. sp. + Ca prot. + Ca disc	2	4.0	13	20.0	5	14
Calcified disc	14	26.0	17	26.0	2	6
Calc. disc and protrusion	10	18.0	12	19.0	9	26
Calc. protrusion	2	4.0	6	9.0	1	3
Osteophytes of adjacent vertebrae	1	2.0	13	20.0	1	3
Ca protrusion + osteophytes	1	2.0	0	0.0	1	3
Calcified disc and osteophytes	1	2.0	2	3.0	0	0
Ventral spondylitis ossificans and no disc change	Not noted by Olsson		11	17.0	6	17

* Not verified by autopsy but by contrast medium and surgery.

thought to be abnormal on spinograms but were normal on autopsy.

The author's data of 64 discs examined by x-ray in 23 dogs autopsied, as well as the 35 discs in 15 dogs established by normal or contrast myelogram and/or by surgery, correspond quite closely to that of Olsson.[13] However, a considerably higher percentage of the discs were found to have no change and a lower percentage were found to have a narrower space between vertebrae. Thirty-seven per cent of the protrusions compared with Olsson's 50 per cent were found to be abnormal in the normal contrast myelogram. However, since multiple lesions were seen in most dogs, the diagnosable protrusions (those causing current symptoms) would be far less than 37 per cent. The author also concurs with Pommer[15] that a calcified nucleus pulposus may have no significance as to herniation or protrusion but merely indicates a degenerative change in the disc.

In a normal contrast roentgenological examination, the following points are listed in summary:

1) Roentgenograms should be taken at least in the lateral and dorsal ventral positions. Occasionally, slightly oblique pictures are helpful.
2) By careful technique and interpretation, 37 per cent of disc protrusions may be noted. Diagnosis of lesion location can be made in some instances by such findings when correlated with neurological examination results.
3) Disc calcification is an indication of degeneration, not necessarily of protrusion (fig. 1).
4) Narrowed intervertebral disc space is infrequently found in protrusion and when found does not always indicate protrusion (fig. 2).

CONTRAST ROENTGENOGRAPHY

Since it was noted that 63 per cent of the protruding discs seen on autopsy were thought to be negative on previous normal contrast spinograms, some aid must be employed for further diagnosis. Contrast myelography has partially, at least, filled that need.

Sicard and Forestier[16] in 1922 made a definite contribution to the diagnosis of this condition by using iodized poppyseed oil. Dandy[8] in 1925 localized a spinal tumor by use of air injection. Since

Fig. 2—PM 121. After thorotrast administration in the cisterna magna, 1-2L disc shows no protrusion, while 4-5L disc demonstrates a slight elevation. These findings were confirmed on autopsy.

the time of this pioneer work in contrast myelography, many workers in the human field have perfected various techniques.

In veterinary literature, there has been very little written on the use of contrast mediums. Brook[8] used lipiodol extensively in his work reported in "The Spine of the Dog." Scattered references to iodized oil used as a result of this work have come from Schnelle,[17] Riser,[16] Boddie,[1] Frauchiger and Fankhauser.[7] Olsson [13] has reported the use of a water-soluble contrast medium called "thorotrast." In his hands, this has given satisfactory diagnostic results but it tended to be toxic unless removed by a forced drainage procedure described by Kubie and Retan.[11]

In the choice of a suitable contrast medium, this author noted a comparison of the various mediums as tabulated by a U. S. naval medical bulletin and reprinted in Bradford and Spurling.[2]

Advantages	*Disadvantages*
PANTOPAQUE	
1) Excellent contrast.	
2) Demonstrates small irregularities.	
3) Easy to aspirate.	
4) Minimal aftereffects.	
5) Minimal evidence after aspiration.	
6) Can examine entire spinal canal.	
LIPIODOL	
1) Excellent contrast.	1) Difficult to aspirate.
2) Demonstrates small irregularities.	2) Aspiration less complete or multiple aspirations are necessary.
3) Moderate aftereffects.	
4) Can examine entire spinal canal.	
AIR	
1) No aspiration necessary.	1) Poor contrast.
2) Moderate aftereffects.	2) Does not demonstrate small irregularities.
3) No evidence of medium.	3) Can not examine entire spinal canal.
THOROTRAST	
1) Excellent contrast.	1) Complicated procedure for drainage.
2) Demonstrates small irregularities.	2) Severe aftereffects.
	3) Residue after drainage.
	4) Can not examine entire spinal canal.

On the basis of the above, most of the research was conducted with pantopaque and some with thorotrast (fig. 1, 2, and 3).

Characteristics of Pantopaque and Thorotrast.—Pantopaque* is a rather recent product developed by Strain *et al.*,[20] Plati *et al.*,[14] and Steinhausen [19] and is used extensively in human myelography in America. It is a mixture of ethyl esters of isometic iodophenylundecylic acids the principal constituent of which is probably ethyl iodophenylundecylate. It is an absorbable oil-type contrast medium of low viscosity. It contains 30.5 per cent of iodine in firm organic combination with a specific gravity of 1.26 at 20 C. This product is a colorless to pale yellow liquid which does not deteriorate on storage, may be sterilized, and is discolored by exposure to sunlight (fig. 3).

Thorotrast** is a water-immiscible suspension or emulsion containing 25 per cent thorium dioxide and a protective colloid of a carbohydrate nature. It is a brownish fluid and should be stored at 60 to 70 F. Thorium dioxide is somewhat radioactive and according to the Council on Pharmacy and Chemistry of the American Medical Association [5] the *alpha* radiation in 25 cc. of thorotrast is equivalent to 0.5 to 1.0 μg. of radium, *beta* radiation is too feeble to be of physiological significance, and *gamma*

* Lafayette Pharmacal Co., Lafayette, Ind.
** Heyden Chemical Corp., New York

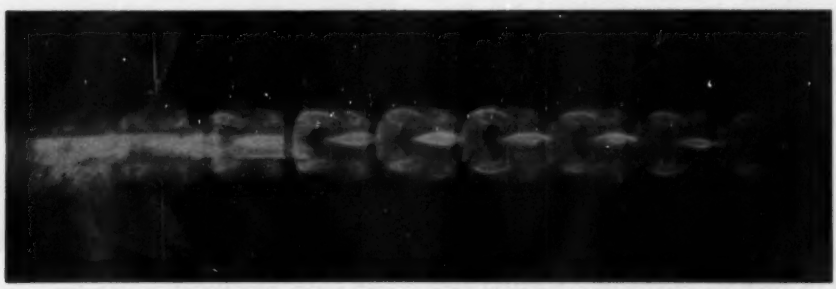

Fig. 3—C26. Dorsoventral view of the pantopaque block at 1-2L. Pantopaque was given in the cisterna magna after the normal contrast findings were negative.

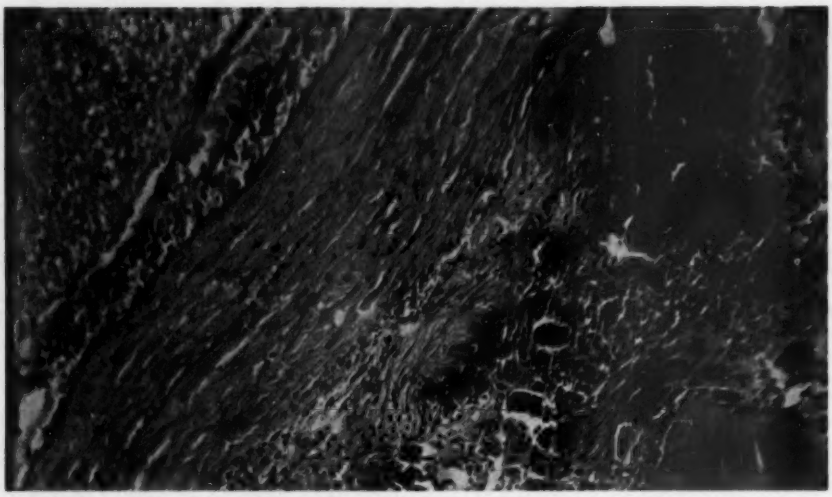

Fig. 5—E42. A characteristic leptomeningitis from subarachnoid administration of pantopaque. From left to right: cord tissue, leptomeningitis, dura mater, and extradural deposit.

radiation in 25 cc. of thorotrast is equivalent to 0.4 μg. of radium (fig. 1, 2).

Toxicity of Pantopaque and Thorotrast.—In both experimental cases and in clinical cases, the subarachnoid administration of pantopaque was found to give little or no clinical reaction. However, a marked rise in w.b.c. in spinal fluid was seen which usually subsided to normal again in two to four weeks. Moreover, it was seen that histopathological sections would reveal a mild fibrosis and thickening of the leptomeninges, especially in the posterior sections of the cord. This reaction as seen by Steinhausen[19] and co-workers was termed a cystic condition of the meninges as a result of a foreign body reaction. Microscopically, the leptomeninges may show round cell infiltration and an abnormal increase in fibrous tissue and fibroblasts (fig. 4, 5).

It would, therefore, be desirable to aspirate pantopaque after the diagnostic procedures have been completed but, according to Steinhausen,[19] the space relations of the cord and spinal canal are such that the medium flows slowly. Practically none can be removed by lumbar puncture.

Thorotrast was used only experimentally in this research except in 1 survival case. It tended to be quite toxic especially if any of the medium migrated into the suboccipital space. As seen from the accompanying table, 5 out of 7 dogs showed marked clinical effects or died in convulsions if the medium was not removed by subsequent forced drainage. Forced drainage as em-

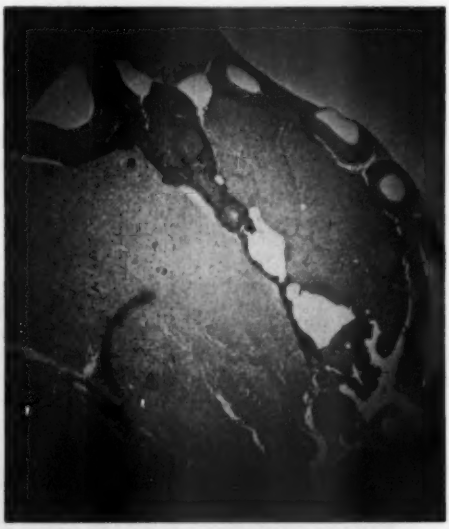

Fig. 4—E3. Leptomeningitis resulting from pantopaque administration into the subarachnoid space. Notice the marked encystment of pantopaque in the leptomeninges (low).

ployed by Kubie and Retan [11] and as used on dogs by Olsson removed a good share of the medium from the subarachnoid space. This drainage, however, seemed to be a laborious procedure and would probably not be practical for a veterinarian to employ.

Thorotrast also causes a marked rise in spinal fluid w.b.c. Histopathological sections reveal a marked phagocytosis of thorium dioxide and a necrotic type of exudate. In some instances this reaction nearly obliterated the meningeal spaces. The fibrotic leptomeningitis occurs much the same as in use of pantopaque (fig. 6).

Summarizing, it might be stated that although pantopaque is slowly absorbed from the subarachnoid space, it may cause a fibrotic leptomeningitis or become encysted. However, clinical effects have not been noted. Thorotrast causes convulsions and death if any material gains access to the suboccipital space. It also causes a fibrotic leptomeningitis as well as an extreme cellular phagocytosis. Both mediums are foreign bodies and the reactions are characteristic of that fact.

Route and Method of Administration.—In human myelography, only the subarachnoid route of injection is pursued. In veterinary medicine, mention of epidural administration has been made by Greene.[6] Olsson evaluates a work by Meyer in which thorotrast was given in the subarachnoid space at the lumbosacral junction. Olsson states that this is a most difficult spot to give subarachnoid injections and that, in analyzing Meyer's roentgenograms, he concluded that the administration was actually made in the epidural space.[13]

The author, after giving injections of thorotrast and pantopaque in both the epidural and subarachnoid space, concluded that subarachnoid administration was far superior. One experiment was conducted in which aluminum foil wads were placed in the spinal canal via trephine openings in the lateral laminae. Of these balls, one measured 4 mm. in diameter and was placed at 2-3L (left side); the other was 8 mm. in diameter and was placed at 4-5L (right side). Then at the 13-1L level, a trephine hole in the lateral lamina was made, 2 cc. of pantopaque was carefully injected epidurally. The dog was tilted at 45 degrees for fifteen minutes and then at 90 degrees for ten minutes with his head up. The resulting spinograms showed unsatisfactory progression of the contrast medium and were of no diagnostic value. Then 2 cc. of pantopaque was given in the subarachnoid space at the same level (13T-

TABLE 2—Toxicity Results with Pantopaque and Thorotrast

Substance used	Dogs (No.)	Route administered	Spinal fluid examined (dogs)	Reaction in spinal fluid	Bromsulfalein [12] and NPN	
Pantopaque	8	Subarachnoid 7	4	Inc. w.b.c. in 24 hours; normal in 3 to 4 wk.; Pandy—0 to 2 plus in all 4 dogs.	Negative	Normal
		Epidural—1	1	No reaction	Negative	Normal
Thorotrast	9	Subarachnoid 8	3	Inc. w.b.c. in 24 hours; normal in 3 to 4 weeks.	10 to 30 % in all 3 dogs; normal in 3 to 4 weeks.	Normal
		Epidural—1	1	No reaction	Negative	Normal

Substance	Clinical effect			Forced drainage	Clinical symptoms
	none	some	marked/death		
Pantopaque					
Subarachnoid	5	1	0	No	Partial inappetance, depression
Epidural	1	0	0	No	None
Thorotrast					
Subarachnoid	3*	2	5	3	Pain, inappetance, depression Death from cerebellar and cerebral meningeal effect.
Epidural	1	0	0	0	None

* All forced drainage: Bromsulfalein—5 mg./kg.—30 min. interval; NPN—Total nonprotein nitrogen.

1L), and when the dog was tilted at 45 degrees for ten minutes very satisfactory diagnostic myelograms were made, showing both aluminum wads at 2-3L and 4-5L levels (fig. 7, 8).

It has also been observed that in epidural routes of administration, one should use larger amounts of mediums than in subarachnoid injections. If given epidurally, the extradural contour of the spinal canal instead of the intradural contour of the spinal cord medulla will be seen. The mediums may flow out along spinal nerve trunks and invade tissue in epidural injections. This can be confusing in interpretation. Interpretation is more difficult at best unless a very large protrusion exists. However, epidural administration of contrast mediums has the advantage of being less toxic to the meninges than subarachnoid injection.

If it be granted that subarachnoid administration is more satisfactory than the epidural route, which injection site is preferable? According to Brook,[3] the only possible location for subarachnoid puncture in the dog is the cisterna magna at the atlanto-occipital junction. However, Cadiot[4] describes subarachnoid puncture at 7L-1S and also at 6-7L. Olsson, whose work was published at the time the author had been independently performing lumbar punctures, reported lumbar subarachnoid punctures at 7L-1S, 6-7L, or even at 5-6L. Olsson's[12] method was to start at 7L-1S and, if not successful there, progress forward to 6-7L. Success was determined if spinal fluid was obtained.

This author readily admits that cisternal punctures are easily done and are relatively safe after a technique has been developed. The technique described by Brock[3] is very satisfactory and is essentially this:

1) Place the dog or cat on his belly and flex the head to a 90 degree angle with the cervical spinal plane.

2) Locate the occipital protuberance and the most prominent palpable part of the wings of the atlas, bisect the midline in its center or slightly behind the center of the line drawn between these points, and direct the needle perpendicular to the cervical axis until the atlanto-occipital space is reached.

The needle must be kept in the midline or blood will be obtained. An 18- to 23-gauge short bevel needle 2½ to 3 in. long is sat-

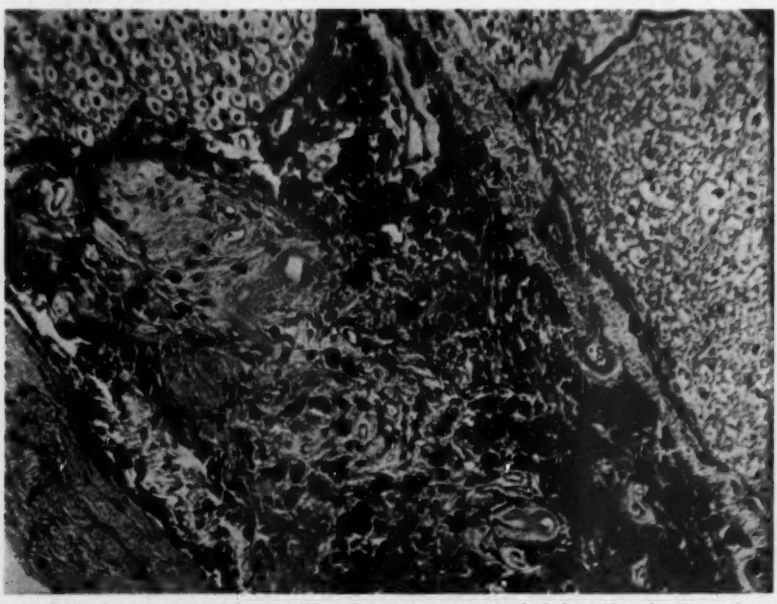

Fig. 6—E36. Leptomeningitis resulting from thorotrast administration in the subarachnoid space. Postadministration period was two months. Note the dark masses of free and phagocytized thorotrast particles and the fibrotic tissue reaction (high dry).

isfactory. The bevel of the needle should be directed caudally and the spinal fluid must be slowly and carefully withdrawn. The amount of contrast medium administered is equal to the amount of spinal fluid withdrawn. Slow injection is necessary in order not to force the fluid out around the needle or through a previous puncture hole. The dog is then tilted from a 30 to 40 degree angle so as to allow caudal migration of the medium. Periodic roentgenological examinations are made in the dorsoventral position (dog on his sternum) and after the lesion is located, a lateral or an oblique exposure is made. This is done under sodium pentobarbital anesthesia. The dog's condition should be constantly checked, since these dogs are often in a weakened condition and are not favorable anesthetic risks.

It is possible to make lumbar punctures and contrast medium injections. The author's experience with these has sometimes been very satisfactory; however, other attempts have led to unfortunate results or failure. The quantity of spinal fluid in this region is rather scanty and often it is difficult to determine whether or not the needle is in the subarachoid space. Also the injection must be made very carefully and slowly or much of the medium will be forced out around the needle, and an epidural administration will result. This injection is often accomplished to verify a cisternal administration, whether the lesion is single or multiple. The method employed by the author and suggested by Monlux [12] is as follows:

1) Tilt dog downward (tail downward), and moderately arch the back. The dog may be in a lateral or a sternal recumbent position.

2) Locate dorsal spinous process of the vertebra desired.

3) Insert 20- to 23-gauge needle at the anterior border of the spinous process, direct it ventral and perpendicularly to the vertebral axis, and the interareual space is felt. The back may have to be arched further at this point in order to make more room for the needle. The dura is carefully punctured, fluid is allowed to drip out of the needle and the injection is made.

The injection site is usually the 6-7L, 5-6L, or the anterior lumbar spaces. The 7L-1S location is uncertain for a subarachnoid injection.

To facilitate movement of the medium when using pantopaque, it is well to periodically flex and massage the back while the dog is maintained in the sternal recumbent position (fig. 3). Lateral pictures should be taken when possible with the dog in the same dorsoventral position so as to get a true picture of the contrast medium on the floor of the subarachnoid space (fig. 1, 2). This will necessitate a movable x-ray tube or a table which will tilt vertically. If this is not possible, make exposures very quickly after changing the dog to a lateral position so that a picture of the floor instead of the lateral side of the subarachnoid space is seen. This is not so important in injections of thorotrast or other water-immiscible mediums, since these agents fill the entire meningeal cavity regardless of position.

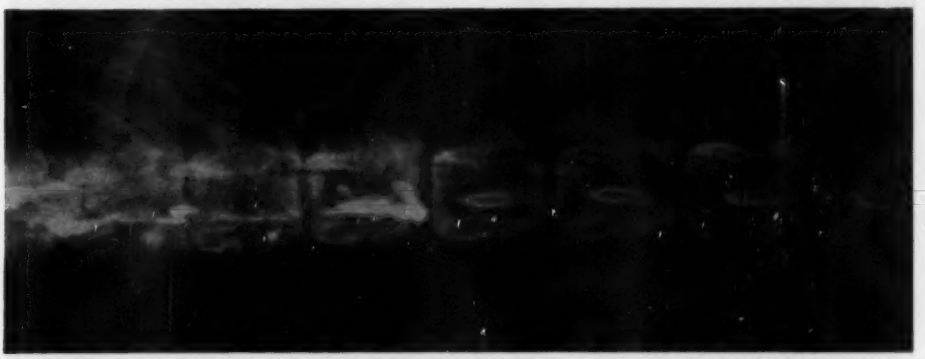

Fig. 7—E16. Pantopaque (2 cc.) was given at the 13T-1L epidural space. The picture was taken after fifteen minutes of tilting the dog (with the head up) at a 45 degree angle. Notice that there is little or no progression of the medium.

INTERPRETATION AND RESULTS OF CONTRAST MYELOGRAPHY

It should be borne in mind that the interpretation of contrast myelography must be correlated with both normal contrast x-ray and neurological examinations. Very frequently, pantopaque will stop its flow about one vertebra from the actual protrusion. This is true especially if a marked extradural necrotic deposit is present from a recent protrusion or prolapse. If an acute disc rupture occurs and a progressive paralysis results, cord edema or extensive subarachnoid hemorrhage might cause an impeded flow of pantopaque and a diagnosis will be impossible. In as much as progression of the medium is slow in the 13T-1L area, it is well to allow more time for its passage at this point.

Contrast myelography was used in 21 dogs suffering with natural protrusions as well as in many experimentally produced protrusions. In nearly every instance, the artificial protrusions were clearly seen with contrast myelography.

Of the 21 natural cases, 8 were entirely negative to any protrusion on normal x-ray pictures and were positive to protrusion on contrast x-ray. Of these 8, 5 were inspected by intraspinal surgery and/or autopsy. Four of these 5 (80 %) were confirmed as indicated by contrast myelography. The other 1 case of the 5 revealed an acute disc rupture, a profuse extradural reaction, and extensive subarachnoid hemorrhage. Here the medium stopped flow at 13T-1L while the causative disc was at the 4-5L disc.

Of the 21 natural disc cases, contrast myelography was employed on 13 cases which showed disc pathology on normal contrast x-ray. Seven cases verified the normal x-ray findings as established upon autopsy or intraspinal spinal surgery. Three contrast myelograms revealed added, or more significant, diagnostic findings that were established on autopsy. The other 3 cases in which a contrast medium was used, did not verify, add, or detract from the normal findings. This was due to technical difficulties of administration. No cases of less significant findings were noted.

Four dogs, which did not have protruding discs, were given contrast mediums. Three of these were negative on normal spinograms, 1 was diagnosed as spondylitis, and the other 2 were nonspecific "old dog" encephalitis. The remaining case showed a total block in the contrast spinogram but was found to have a medulloblastoma on histopathological section; however, the tumor lesion was correctly located by the use of contrast medium.

SUMMARY OF MYELOGRAPHIC METHODS

The suggestions for myelography methods both for normal and contrast mediums have been described. The contrast medium considered to be most practical for the veterinarian was pantopaque although thoro-

Fig. 8—E16. Pantopaque (2 cc.) was given in the subarachnoid space at the 13T-1L site. The dog was tilted ten minutes at a 45 degree angle. The radiogram demonstrates the compressions caused by objects placed in the canal at the 2-3L and 4-5L disc locations.

trast is more easily used and the results are more readily interpreted. Since thorotrast is more toxic and must be drained by a laborious procedure, it has little place in veterinary practice. Because of the relatively small subarachnoid space in the dog which results in a rather slow progression of pantopaque in this area, the findings must be interpreted only when correlated with results of normal x-ray and neurological examinations. With experience, many diagnoses, especially of the recurring individual lesion variety, can be made on normal contrast x-ray pictures. In these cases, never give a contrast medium and subject the dog to meningeal reactions associated with these foreign substances unless it is absolutely necessary. However, when properly used, contrast medium introduced in the subarachnoid space is of extreme value in establishing the correct diagnosis of protrusion site in select cases.

References

[1] Boddie, G. F.: Differential Diagnosis of Canine Paraplegia, a Clinical and Radiological Study. Vet. Rec., *61*, (1949): 511-515.

[2] Bradford, F. K., and Spurling, R. G.: The Intervertebral Disc. 2nd ed. Thomas, Springfield, Ill., 1945.

[3] Brook, G. B.: Experimental and Clinical Studies of the Spine of the Dog. Wood, Baltimore, Md., (1936): 12, 35.

[4] Cadiot, P. J., Lesbouyries, G., and Ries, J. N.: Traite de Medecine de Animaux Domestiques. Vigot Frerere, Paris, (1925): 535.

[5] Council on Pharmacy and Chemistry. Thorotrast. J. Am. M. A., *99*, (Dec. 24, 1932): 2183.

[6] Dandy, W. H.: A Sign and Symptom of Spinal Cord Tumors. Arch. Neurol. and Psychiat., *16*, (1926): 435.

[7] Frauchiger, E., and Fankhauser, R.: Die Nervenkrankheiten unserer Hunde. Schmid & Cie, Bern., (1949): 49.

[8] Greene, J. E.: Surgical Intervention for Paraplegia due to Herniation of the Nucleus Pulposus. North Am. Vet., *32*, (1951): 411-412.

[9] Hoerlein, B. F.: Intervertebral Disc Protrusions in the Dog. II. Symptomatology and Clinical Diagnosis. Am. J. Vet. Res., *14*, (1953):

[10] Hoerlein, B. F., and Greene, J. E.: The Bromsulfalein Liver Function Test as an Aid in the Diagnosis of Canine Hepatosis. North Am. Vet., *31*, (1950): 662-665.

[11] Kubie, L. S., and Retan, G. M.: Forced Drainage of the Cerebrospinal Fluid. J. Am. M. A., *101*, (1933): 354.

[12] Monlux, W. S.: Personal communication.

[13] Olsson, Sten-Erik: On Disc Protrusion in Dog (Enchondrosis Intervertebralis). Acta Orthopedica Scand. Supplementum No. VIII, Ejnar Munksgoard, Copenhagen, 1951.

[14] Plati, J. T., Strain, W. H., and Warren, S. L.: Iodinated Organic Compounds as Contrast Media for Radiographic Diagnosis. II. Ethyl Esters of Iodinated Straight and Branched Chain Phenyl Fatty Acids. J. Am. Chem. Soc., *65*, (1943): 1273.

[15] Pommer, A.: Tierheilk, u. Tierzucht., (Stang. u. Wirth), *11*, (1937): 134.

[16] Riser, W. H.: Posterior Paralysis Associated with Intervertebral Disc Protrusion in the Dog. North Am. Vet., *27*, (1946): 633-642.

[17] Schnelle, G. B.: Radiology in Canine Practice. North American Veterinarian, Evanston, Ill., (1950): 128.

[18] Sicard, J. A., and Forestier, J.: Methods general d'exploration radiologique par l'huile iodee (lipiodol). Bull. et mém. Soc. med. d. hôp. de Paris, *46*, (1922): 463-469.

[19] Steinhausen, T. B., Dungan, C. E., Furst, J. B., Plati, J. B., Smith, S. W., Darling, A. P., Wolcott, E. C., with Warren, S. L., and Strain, W. H.: Iodinated Organic Cpds. as Contrast Media for Radiographic Diagnoses. III. Experimental and Clinical Myelography with Ethyl Iodophenylundecylate (Pantopaque). Radiology, *43*, (1944): 231.

[20] Strain, W. H., Plati, J. T., and Warren, S. L.: Iodinated Organic Compounds as Contrast Media for Radiographic Diagnosis. I. Iodinated Aracyl Eesters. J. Am. Chem. Soc., *64*, (1942): 1436.

The Determination of the Lethal Dose 50/30 of Total Body X-Radiation for Dogs

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TOTAL BODY radiation has been defined as the exposure of the entire body surface to ionizing radiation in such a fashion that a dose distribution as uniform as possible is obtained within the body.

The primary objective of this study was to determine the $l.d._{50/30}$ of total body radiation, *i.e.*, that dose which, within thirty days, will kill 50 per cent of the dogs exposed.

The source of the ionizing radiation in this study was the 2,000 kv.p. x-ray machine generator at the Naval Ordnance Laboratory at White Oak, Md. The comparability of total body x-radiation to ionizing radiation from a nuclear explosion and the use of this tool in the laboratory in lieu of a nuclear explosion, has been established.[1,2]

MATERIALS AND METHODS

The characteristics of the 2 million volt industrial x-ray machine at the Naval Ordnance Laboratory have been described previously.[3] Since depth-dose measurement indicated that approximately 50 per cent of the air dose was delivered to the median saggital plane of the animal, one half of the total dose was delivered to each side of the animal to achieve a uniform dose distribution throughout the body.[4] The animals were irradiated at a 2-m. radius from the tube. The animal exposure cages were built of wood, and the plywood sides containing numerous holes for ventilation were curved so as to form the arc of a circle with a radius of 2 m. The physical data on the x-ray machine are given in table 1.

The machine was calibrated with a 100 r. Victoreen ionization chamber before irradiating the animals in each series. The output of the machine remained constant at a radius of 2 m. and a vertical distance of from 88 to 108 cm. from the floor. Irradiation of the dog was accomplished within this field.

The dogs used in this study were of the Walker Foxhound variety. They varied in weight from 30 to 45 lb. and in age from 2 to 6 years. Almost all of them were males and they were in good flesh, although some of them were moderately parasitized with hookworms and whipworms.

All of the animals were irradiated in the same position and in such a fashion that the sides of the animals touched the sides of the rigid cages. After one half of the dose was given, the position of the animals was reversed. The animals were held in their wooden cages by means of wooden dowels, so it can be said they received an equal dose distribution to each side. Specific data relative to radiation dosage and mortality data are given in tables 2 and 3.

Necropsy was performed immediately on all animals that died. Gross studies only were carried out on those animals in the 3,000, 1,000, and 800 r. series. Gross and microscopic studies were per-

MORTALITY RATE AS A FUNCTION OF DOSE - GROUP I

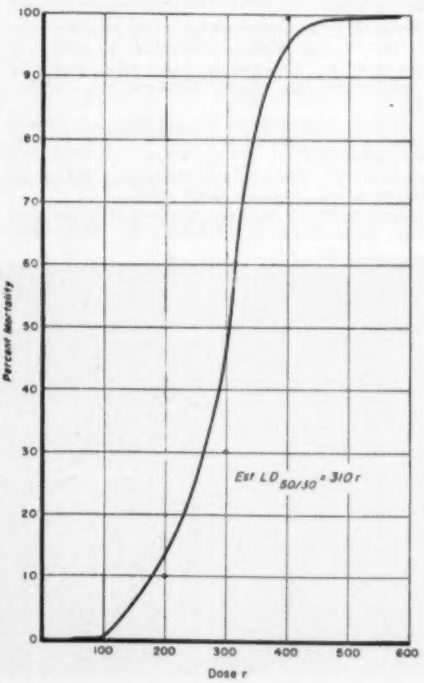

Fig. 1—Graphic representation of the mortality data.

This study was performed at the Naval Medical Research Institute, Bethesda, Md., and the Armed Forces Institute of Pathology, Washington, D. C.

The author is indebted to the staff of the Naval Medical Research Institute, especially Commander E. P. Cronkite, at the National Naval Medical Center, Bethesda, Md., for valuable assistance in the irradiation of the animals, and to Commander R. Lee, M.S.C., U.S.N., for the probit analysis of the data.

TABLE 1—Physical Data on X-Ray Generator

Kilovolt peak	2,000
Milliamperes	1.50
Target-skin distance	2 meters
Inherent filter	6.3 mm. Fe
Added filter	None
Half-value layer	4.3 mm. Pb

TABLE 2—Survival Time After Total Body X-Radiation

Dose (r.)	Range of survival time (days)	Mean survival time (days)
3,000	3 to 4	3.3
1,000	3 to 9	6.6
800	9 to 17	11.7
600	4 to 21	12.2
500	7 to 12	9.8*
400	6 to 23	16.2
300	20 to 30**	.....
200	11 to 30**	.....

* Epizoötic of distemper; ** thirty-day survivors.

TABLE 3—Mortality Data

Dose (r.)	No. Anim.	No. dead (30 days)	% Mortality (30 days)
3,000	5	5	100
1,000	8	8	100
800	4	4	100
600	10	10	100
500	10	10	100
400	10	10	100
300	10	3	30
200	10	2	20

TABLE 4—Mortality Data on Additional Dogs

Dose (r.)	No. Anim.	No. dead (30 days)	% Mortality (30 days)
450	4	4	100.0
400	19	17	89.5
350	8	4	50.0
300	25	6	24.0
200	11	3	27.3

formed on all other animals. The results of the pathological studies will be reported in a subsequent paper.

RESULTS

Bilateral total body x-radiation in doses of 3,000, 1,000, 800, 600, 500, and 400 r. produced 100 per cent mortality in the dogs used in this experiment A dose of

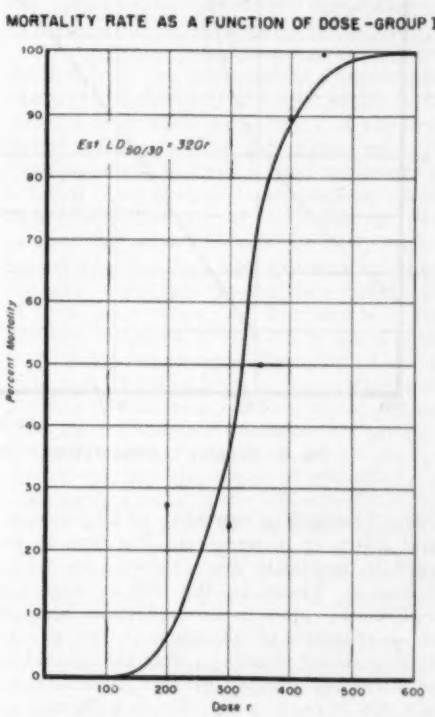

Fig. 2—Graphic representation of the mortality data on additional dogs.

TABLE 5—Mortality Rate as a Function of Dose

Dose	Log dose	No. of animals exposed	No. of animals dead	Mortality (%)	Empirical probits	Weighted probits $\bar{n}$ (y)	Calculated mortality (%)	Standard error log dose (r.)	Fiducial limits (95% confidence) (r.) high	(r.) low
450	2.65321	4	4	100.0	.....	6.01559	84.4	0.09044	873.0	232.0
400	2.60206	19	17	89.5	6.2536	5.68733	75.1	0.06899	663.1	241.3
350	2.54407	8	4	50.0	5.0000	5.29579	61.6	0.05206	512.5	239.1
300	2.47712	25	6	24.0	4.2937	4.85453	44.2	0.05254	440.9	204.1
200	2.30103	11	3	27.3	4.3962	3.6948	9.6	0.12365	494.9	80.8
315.6	2.49918	.....	.....	.....	$LD_{50/30}$	.....	.....	0.04970	454.3	219.3

$\bar{n}$
$y = -11.47848 + 6.59355x$
S.E.(a) = 0.3245
S.E.(b) = 3.5440

$x_5^2 = 11.48045$ (P about .01)
$LD_{50/30} = 2.49918 \pm 0.04970$
$LD_{50/30} = 315.6$

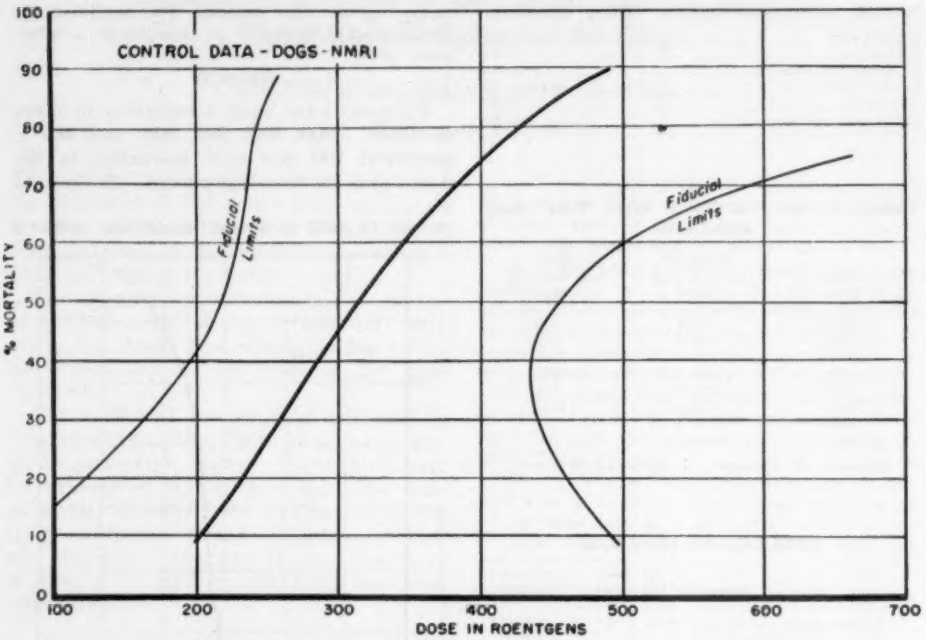

Fig. 3—Graphic representation of the mortality rate as a function of dose.

300 r. resulted in mortality of 30 per cent, and 200 r. of 20 per cent. The data in regard to mortality are presented in tables 2 and 3. Those on the 500 r. dogs are legitimately open to some question, because of an epizoötic of distemper in this group. It is believed, however, that the mortality rate was not affected by this circumstance, nor did it exert a significant influence on the $l.d._{50/30}$. A free hand drawing of the line resulting from the graphic representation of the mortality data (fig. 1) yields an estimated $l.d._{50/30}$ of 310 r. It became apparent at this time that it would be desirable to irradiate more animals in the 200 to 500 r. range to obtain more points on the line and thus a closer $l.d._{50}$. Mortality data on the additional dogs are presented in table 4. A free hand line of these data yields an estimated $l.d._{50/30}$ of 320 as seen in figure 2. A statistical analysis of these data by means of probits yields an $l.d._{50/30}$ of 315.6 r. These data are presented in table 5 and figure 3.

SUMMARY AND CONCLUSIONS

Groups of dogs of the Walker Foxhound variety were exposed to doses of bilateral total body x-radiation varying from 3,000 to 200 r. The source of the radiation was a 2,000 kv.p. x-ray generator. The 50 per cent lethal dose for thirty days ($l.d._{50/30}$) was found to be 315.6 r. The mortality data have been presented in accompanying tables and figures.

References

[1] Gleiser, C. A.: The Sequence of Pathologic Events in Dogs Exposed to an $LD_{100/30}$ Dose of Total Body X-radiation. In press.

[2] Tullis, J. L., and Warren, S.: The Response of Tissue to Total Body Irradiation. Am. J. Path., *25*, (1949): 829-851.

[3] Chambers, F. W., Jr., Morgan, J. E., and Istock, J. T.: Output Characteristics of a Commercial X-Ray Generator at 2,000 KVP. Naval Medical Research Institute Project NM 006.012.08.26, Dec. 7, 1949, National Naval Medical Center, Bethesda, Md.

[4] Tullis, J. L., Chambers, F. W., Jr., Morgan, J. E., and Zeller, J. H.: Mortality in Swine and Dose Distribution Studies in Phantoms Exposed to Super Voltage Roentgen Radiation. Am. J. Roentgenol., Radium Therapy and Nuclear Med., *4*, (1952): 620-627.

The Sequence of Pathological Events in Dogs Exposed to a Lethal Dose 100/30 of Total Body X-Radiation

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TOTAL body radiation is defined as the exposure of the entire body surface to ionizing radiation so as to obtain as uniform a dose distribution within the body as possible. Such an exposure, of necessity, is bilateral.

The concept of radiation injury is not a new one. Wolbach,[1] in a classic piece of work in 1909, described in detail the changes produced in skin and collagen by radiation injury. The concept of total body radiation, however, is relatively new, having been ushered in by the atomic age and, more specifically, by the use of atomic weapons. It differs from other forms of radiation injury in its acuteness and distribution. Therapeutic radiation is acute but is confined generally to a small area. Chronic radiation injury is caused by repeated exposure of some portion of the body surface to ionizing radiation, as the hands of pioneer radiologists. These differences in the manner and rate of exposure are stressed because the tissue reactions induced by these different methods of exposure to ionizing radiation are dissimilar. Changes may be produced in relatively radioresistant tissues by massive focal doses of ionizing radiation.

It is a well-established fact that tissues and cells react in a characteristic manner to ionizing radiation.[2,3] If the reaction is prompt, the tissues are called radiosensitive. If the reaction is delayed, or even absent, the tissues are relatively radioresistant. Radiosensitivity appears to be a function of the rate of mitosis, that is, the tissues with high mitotic activity, such as lymphoid and hematopoietic, appear to be the most radiosensitive. The reaction can be measured by the morphological changes that are produced, but the mechanism of the production of these morphological changes is not as yet clearly understood.

The literature contains numerous reports dealing with the pathological changes produced by acute and chronic exposure to ionizing radiation. Knowledge of the very early changes and the progression of changes resulting from a single exposure to a lethal dose of total body ionizing radiation is not as abundant.[4-8] It was the purpose of this work to study the early pathological changes and the sequence of these changes in animals exposed to a lethal dose of total body radiation. The source of the ionizing radiation in this study was a 2,000 kv.p. x-ray generator. While the effects of total body x-radiation are not exactly comparable to radiation from an atomic explosion, the pathological changes that are produced are similar enough to permit the use of this tool for the study of the effects of ionizing radiation in the laboratory.[9,10] Actually, any source of ionizing radiation that will achieve ionization within the tissues could be used, because the biological effects produced are essentially similar.[5,11,12]

An $l.d._{100/30}$ means a dose that will kill all the animals exposed within thirty days. The air dose of x-radiation used in the study was 600 r.

MATERIALS AND METHODS

The x-ray generator used was the 2 million volt industrial x-ray machine at the Naval Ordnance Laboratory, White Oak, Md. The physical data on the generator as used in this experiment are presented in table 1. More complete details are published elsewhere.[12]

Since the depth-dose measurements indicated that approximately 50 per cent of the air dose was

TABLE 1—Physical Data of X-Ray Generator

Kilovolt peak	2,000
Milliamperes	1.50
Target-skin distance	2 meters
Inherent filter	6.3 mm. Fe
Added filter	None
Half-value layer	4.3 mm. Pb
50 % depth dose in Masonite	14 cm.
Output	15 r./minute ± 2 %

This study was performed at the Naval Medical Research Institute, Bethesda, Md., and the Armed Forces Institute of Pathology, Washington, D. C.

The author is deeply indebted to Brig. Gen. Elbert DeCoursey, M.C., director, and Capt. Arthur W. Eaton, Jr., M.C., U.S.N., formerly deputy director, of the Armed Forces Institute of Pathology, for guidance and advice during the period of study. The work could not have been accomplished without the very capable assistance of the technical staffs of the Naval Medical Research Institute and the Armed Forces Institute of Pathology.

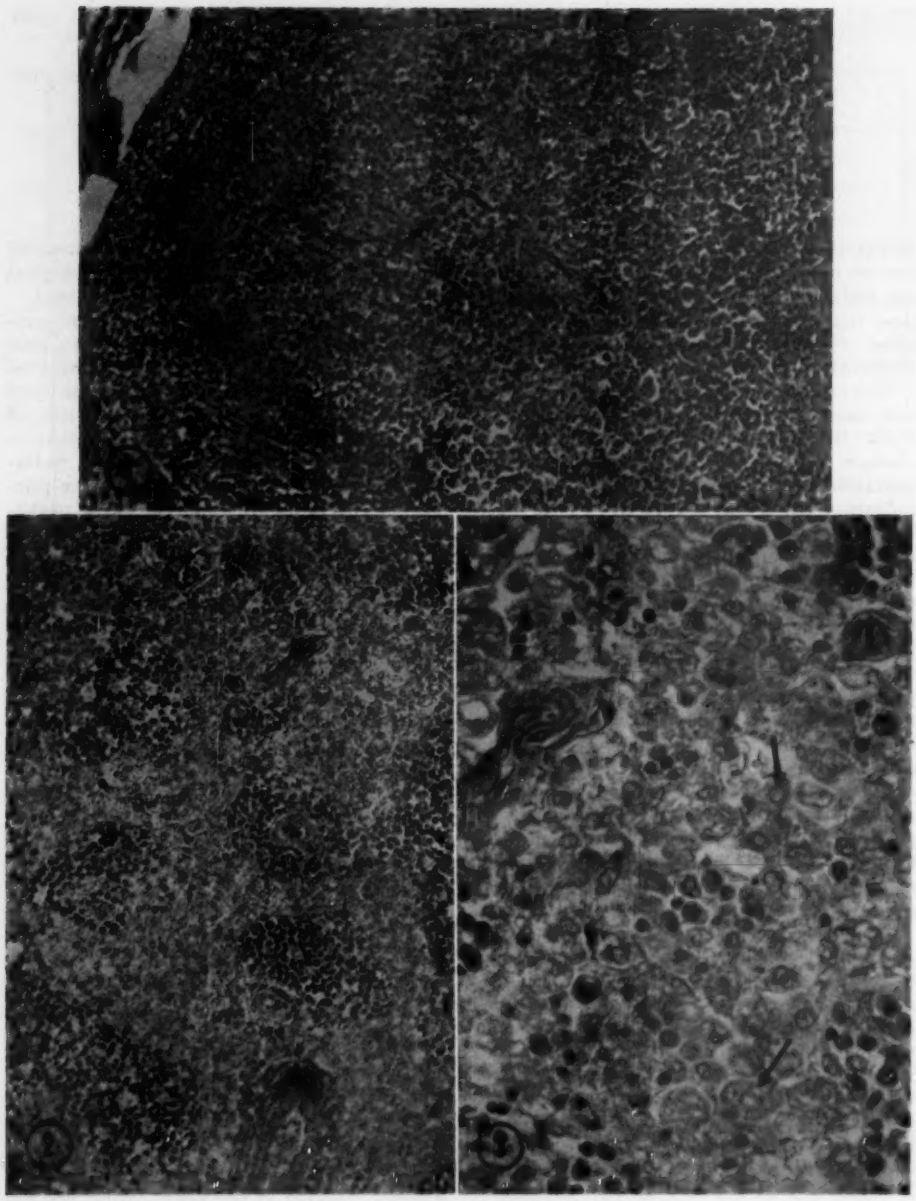

Fig. 1—Lymph node, one hour after irradiation: nuclear debris in follicle. x 140. AFIP 248129.

Fig. 2—Lymph node, one hour after irradiation: medullary sinuses are packed with erythrocytes; plasma cells already are noticeable and erythrophagocytosis is present. x 140. AFIP 248129.

Fig. 3—Lymph node, one hour after irradiation: erythrocytes and erythrophagocytosis in medullary sinus. x 440. AFIP 248129.

delivered to the median saggital plane of the animal, one half of the total dose was delivered to each side of the animal to achieve uniform dose distribution throughout the body.[14] The output of the generator being 15 r. per minute, the animals were irradiated for twenty minutes on one side for a dose of 300 r., turned around and irradiated for twenty minutes on the other side for 300 r.

The animal exposure cages were built of wood, with plywood sides containing numerous holes for ventilation. The sides of the cages were curved so as to form an arc of a circle with a 2-m. radius.

Eighteen adult male dogs of the Walker Foxhound variety ranging in age from 2 to 6 years and in weight from 30 to 45 lb. were irradiated in the manner described. All were in good flesh and appeared to be healthy although some were found to be moderately infested with intestinal parasites. The dogs were serially destroyed by the intravenous injection of lethal doses of sodium pentobarbital in accordance with table 2.

Complete autopsies were performed and the gross, and microscopic pathological changes were studied as a function of time after irradiation. Specimens of tissue were fixed in 10 per cent buffered formalin and prepared for sectioning in the usual manner. Sections were cut at 5 μ and stained with hematoxylin and eosin and Giemsa's stain.

RESULTS

Clinical Course.—Many of the dogs vomited during the first 24-hour postirradiation period. Most of them showed varying degrees of inactivity and malaise during the immediate postirradiation period. Subsequently, they remained in good health. The last dog on the schedule, however, died before the time appointed for autopsy. At no time during the seven-day period did any of the animals exhibit any outward signs of the hemorrhagic syndrome of radiation.

Gross Autopsy Observations.—The changes observed at autopsy were minimal. Many of the animals showed no gross lesions. There was little indication at autopsy that all of these animals would have died within thirty days, probably within twenty days, had they been allowed to live.

Edema of some of the superficial body lymph nodes was observed as early as nine hours after irradiation. Isolated hemorrhagic lymph nodes were observed in dogs autopsied at thirteen hours, twenty-nine hours, and fifty-six hours. Generalized edema of lymph nodes was observed sixty-eight hours after irradiation. Numerous hemorrhagic nodes were observed 104 hours after irradiation. At 164 hours, dog 142, which had died several hours prior to the scheduled time of autopsy, showed numerous diffuse hemorrhages throughout its body.

Redness of the mucosa of the small intestine was observed occasionally, but it was difficult to evaluate in view of the parasitic infestations encountered. This applies as well to the occasional small isolated submucous hemorrhage in the small intestine.

Histopathological Observations.—Histological evidence of ionizing radiation injury was confined to the most radiosensitive tissues, namely, the lymphoid, hematopoietic, intestinal, and genital tissues.

Lymphoid Tissues.—The changes in the lymphoid tissues were observed in all lymphatic elements found in lymph nodes, spleen, tonsil, thymus, and solitary and aggregated follicles of the intestinal wall. The changes in the thymus did not appear as promptly as in the lymph nodes, nor were they as prominent. They were also difficult to evaluate because of the atrophic changes that occur normally in the thymus of the adult dog.

Immediate degenerative changes within the lymphoid tissues were observed even in the first dog, destroyed one hour after irradiation. The lymphocyte destruction in this dog was great and was evidenced by the severe pyknosis and the large amount of nuclear debris within the follicles. These degenerative changes were rapidly progressive and the nuclear debris within the follicles reached a peak at six hours after

TABLE 2—Serial Sacrifice Schedule for Dogs Given 600 r, Total Body X-Radiation

Dog No.	Time destroyed after irradiation (hours)
125	1
126	3
127	6
128	9
129	13
130	21
131	29
132	36
133	45
134	56
135	68
136	80
137	92
138	104
139	116
140	128
141	140
142	164

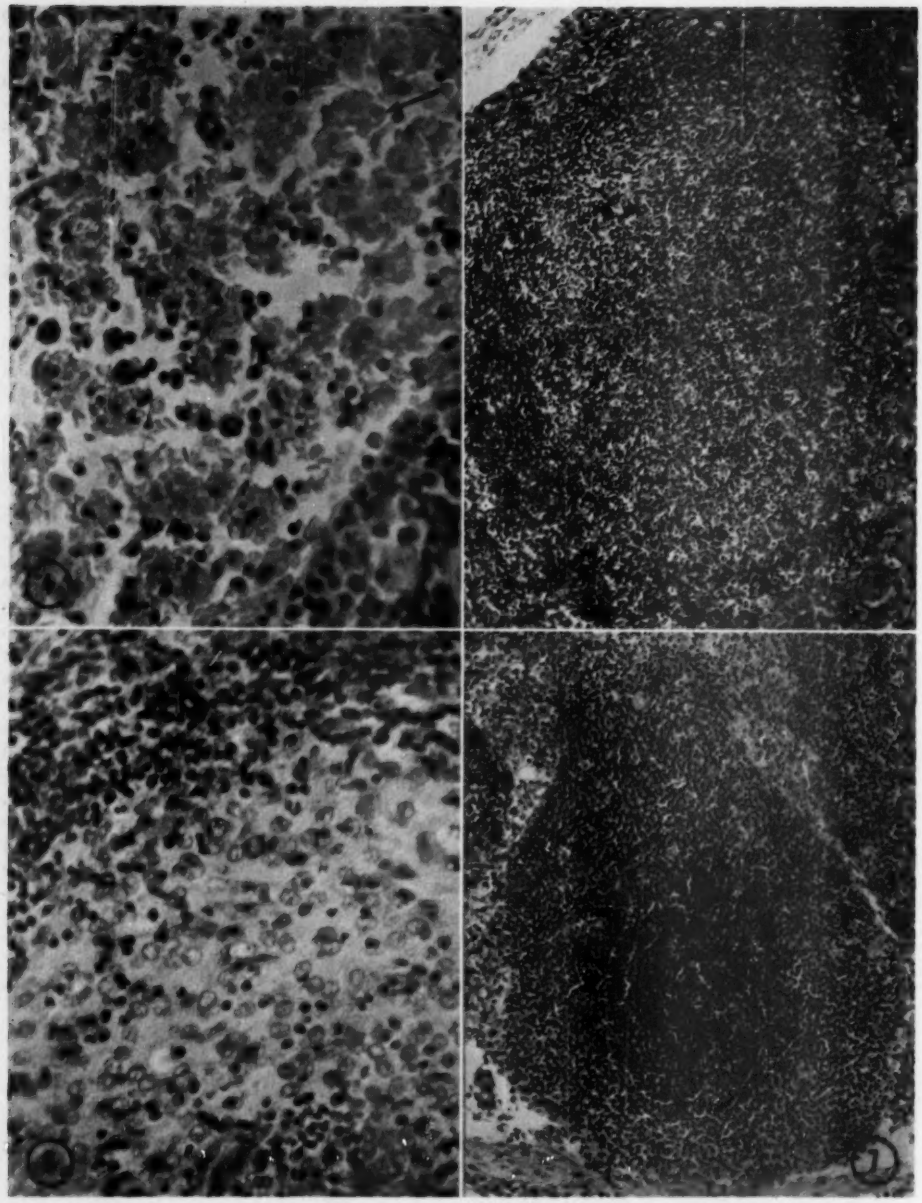

Fig. 4—Lymph node, three hours after irradiation: erythrocytes and erythrophagocytosis in medullary sinus. x 325. AFIP 248130.

Fig. 5—Lymph node, six hours after irradiation: nuclear debris in follicle. x 137. AFIP 248131.

Fig. 6—Lymph node, twenty-nine hours after irradiation: follicle cleared of nuclear debris. x 300. AFIP 248135.

Fig. 7—Lymph node, eighty hours after irradiation: repopulation of follicle. x 105. AFIP 248140.

irradiation, when phagocytes had already begun to remove the debris. Neutrophils were also present, probably to aid in the phagocytosis. Only a rim of large lymphocytes remained around the follicles in the lymph nodes at this time. Nine hours after irradiation many neutrophils and some eosinophils were observed in the follicles and sinuses of the nodes. Twenty-one hours after irradiation most of the nuclear debris had been removed from the follicles, which were composed only of reticular cells. A scanty repopulation of some of the follicles was observed as early as forty-five hours after irradiation. A slow gradual repopulation of the follicles and medullary cords was apparent throughout the remainder of the series. A few mitotic figures were present in some of the follicles 104 hours after irradiation. The repopulation by lymphocytes was clearly evident 116 hours after irradiation.

Striking changes in the sinuses persisted throughout the entire series. In dog 125, destroyed at one hour, there was a tremendous aggregation of actively phagocytic cells of the reticuloendothelial variety. These cells contained pagocytosed erythrocytes in addition to nuclear fragments of lymphocytic nuclei. Dilated sinuses appeared to be filled with these erythrocyte-laden phagocytes and small clumps of aggregated erythrocytes. This erythrophagocytosis was observed throughout the entire series. Phagocytes containing blood pigment were first seen in dog 128, destroyed nine hours after irradiation. In this animal, neutrophils and eosinophils in the sinuses were most numerous. They were seen in smaller numbers throughout most of the series. The neutrophils were much more numerous than the eosinophils. Plasma cells were observed in the sinuses as well as in the follicles and medullary cords soon after irradiation and persisted in small numbers throughout the series.

Another change observed in the sinuses was swelling of the perivascular collagen, first observed twenty-one hours after irradiation and inconstantly throughout the remainder of the series.

Similar changes had occurred in the lymphatic elements of the spleen. The immediate and progressive lymphocyte destruction was characterized by cytolysis, nuclear pyknosis, and fragmentation. Congestion of the splenic sinusoids obscured somewhat the phagocytes laden with erythrocytes and pigment. Large multinucleated cells, which were assumed to be megakaryocytes, appeared within the sinusoids in animals destroyed at longer intervals.

Bone Marrow.—The first microscopic evidence of change within the bone marrow appeared nine hours after irradiation when there was a moderate decrease in erythroid elements, which became more apparent at thirteen hours. At twenty-one hours, the myeloblasts appeared fewer in number. At twenty-nine hours, the diminution of erythroid and myeloid elements was clearly apparent. Degenerative changes within the nuclei and cytoplasm of the myeloblasts and megakaryocytes became obvious at this time, whereas previously such changes had been questionable. There were clumping and fragmentation of the nuclear material and alterations in the staining characteristics of the cytoplasm. The mature erythrocytes, granulocytes, and megakaryocytes were not reduced in number, although the cellularity of the marrow was definitely decreased.

At forty-five hours, dilatation and engorgement of the sinusoids were prominent and the cellularity of the marrow was greatly diminished. No erythroid elements could be recognized; myeloblasts were much reduced in number, and mature myelocytes somewhat less. The megakaryocytes persisted; in fact, it appeared that there was a relative increase in their number.

At sixty-eight hours, megakaryocytes persisted and eosinophils and plasma cells had increased in number, but the cellularity of the marrow was still declining.

At eighty hours, there was a preponderance of eosinophils and plasma cells. There were also numerous degenerating neutrophils which, because of their pyknotic nuclei and eosinophilic cytoplasm, resembled eosinophils. Marked degenerative changes had taken place in the nuclei of the persisting megakaryocytes.

At ninety-two hours, the eosinophils and plasma cells were fairly numerous. There was a decrease in the number of megakaryocytes, and deposition of a fibrinoid material within the sinuses.

The cellularity of the marrow declined even further at 116 hours, with only the mature erythrocytes and plasma cells per-

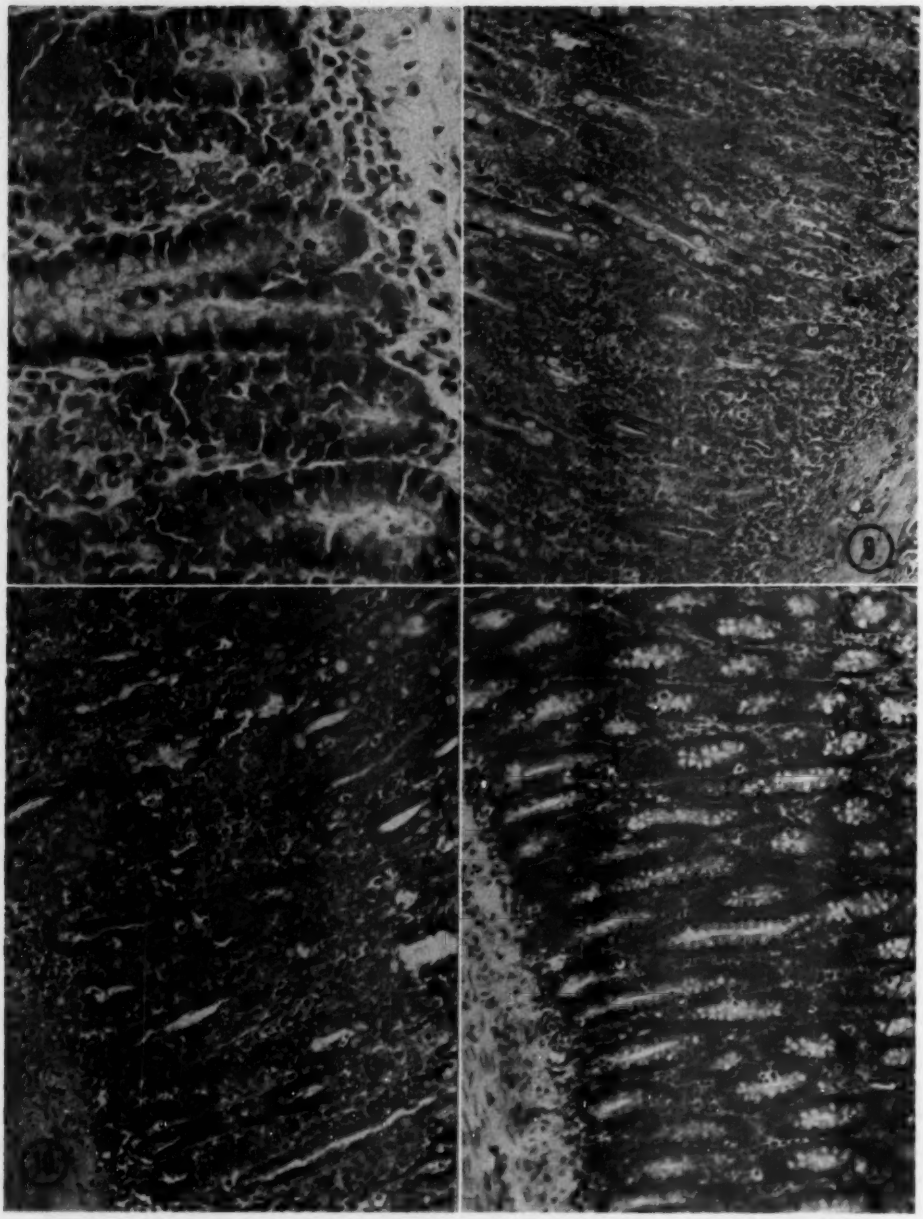

Fig. 8—Duodenum, one hour after irradiation: cessation of mitosis, nuclear degeneration in glandular epithelium. x 340. AFIP 248129.

Fig. 9—Duodenum, twenty-one hours after irradiation: increase in mucous cells. x 137. AFIP 248134.

Fig. 10—Jejunum, twenty-nine hours after irradiation: marked degeneration and atrophy of glandular epithelium. x 137. AFIP 248135.

Fig. 11—Jejunum, 140 hours (six days) after irradiation: regeneration of glandular epithelium. Numerous mitoses. x 105. AFIP 248145.

sisting in large numbers. The marrow was hemorrhagic at 164 hours.

Gastrointestinal Tract.—Significant microscopic lesions were not observed in the stomach. Occasional necrosis of the glandular epithelium was difficult to interpret.

The most significant changes in the gastrointestinal tract were observed in the small intestine, particularly the duodenum and jejunum. There was an immediate arrest of all mitotic activity in the glandular epithelium. Resumption of mitotic activity was first observed thirteen hours after irradiation and became more apparent throughout the series. Degeneration and necrosis of the deep glandular epithelium were noted three hours after irradiation. These changes consisted of nuclear swelling, pyknosis, and rhexis, and change in the staining characteristics of the cytoplasm. The cytoplasm frequently appeared to be vacuolated. The glands became atrophic, their continuity was disrupted, and their content of mucous cells increased. Sloughing of the epithelium of the villous tips was also observed. The capillaries within the villi contained aggregates of erythrocytes, or what perhaps might be called agglutinative thrombi. At fifty-six hours, plasma cell infiltration into the substantia propria was prominent. Repair and regeneration of the glandular epithelium were striking. Changes in the lymphatic elements have already been described.

Testes.—The microscopic changes in the testes were not as regular and constant as the changes in the lymphatic and hematopoietic tissues. Variations appeared from animal to animal, in different tubules in the same animal, and even perhaps in the same tubule if, as has been suggested, the normal cycle of spermatogenesis progresses rhythmically along the tubules in a wavelike manner.[15] Thus, it is entirely possible that adjacent tubules may show quite dissimilar histological pictures. Perhaps only the tubules actively engaged in spermatogenesis are injured by ionizing radiation.

The first detectable change in the tubules was observed at nine hours when there was vacuolation and a peculiar crescentic clumping of the chromatin in many of the nuclei of the spermatogonia. There appeared to be a definite reduction of spermatogenesis at fifty-six hours. Spermatids and spermatozoa were still present, but many of them seemed not to be viable. At sixty-eight hours, this decrease was more apparent and also included the spermatogonia, many of which continued to exhibit vacuolation of the nuclei. At eighty hours, the nuclear vacuolation of spermatogonia was extreme. Some spermatozoa were observed in the tubules but practically no spermatids.

Frank atrophy of the tubules was recognized in the dog destroyed at ninety-two hours. Only Sertoli cells remained in most of the tubules. It is difficult to evaluate this observation properly, because the testes of the subsequent animals were not in such an advanced state of atrophy; in fact, active spermatogenesis, even though greatly reduced, was present in some of the tubules. It is believed that some other factor was concerned in the degree of atrophy of the tubules in the testis of this animal, possibly a very small neoplasm, such as Sertoli cell tumor, that was missed at autopsy.

Definite aspermia was present throughout the remainder of the series, although some spermatogenesis was observed in the last 2 dogs at 140 and 164 hours after radiation. Sertoli cells and interstitial cells proved to be radioresistant and suffered no change, neither were changes observed in the epididymis and prostate.

Endothelium.—Changes in the endothelium of arterioles have been described in radiation injury resulting from total body radiation. Endothelial swelling and distortion were observed in numerous arterioles throughout the series. Such changes are difficult to differentiate from those occurring in the endothelial lining of arterioles as results of normal physiological activity and preparation of the sections. This same reasoning applies to any changes in the thickness of the arteriolar wall without an obvious increase in its cellularity. Using conventional methods, it was impossible to note any changes in the capillary endothelium, but it is suspected that damage to capillary endothelium may occur early and may be partially responsible for the erythrophagocytosis seen in the lymph nodes and the clumping of the erythrocytes observed in the capillaries of the intestinal villi, the alveoli, and the glomeruli. These clumped erythrocytes resemble minute agglutinative thrombi.

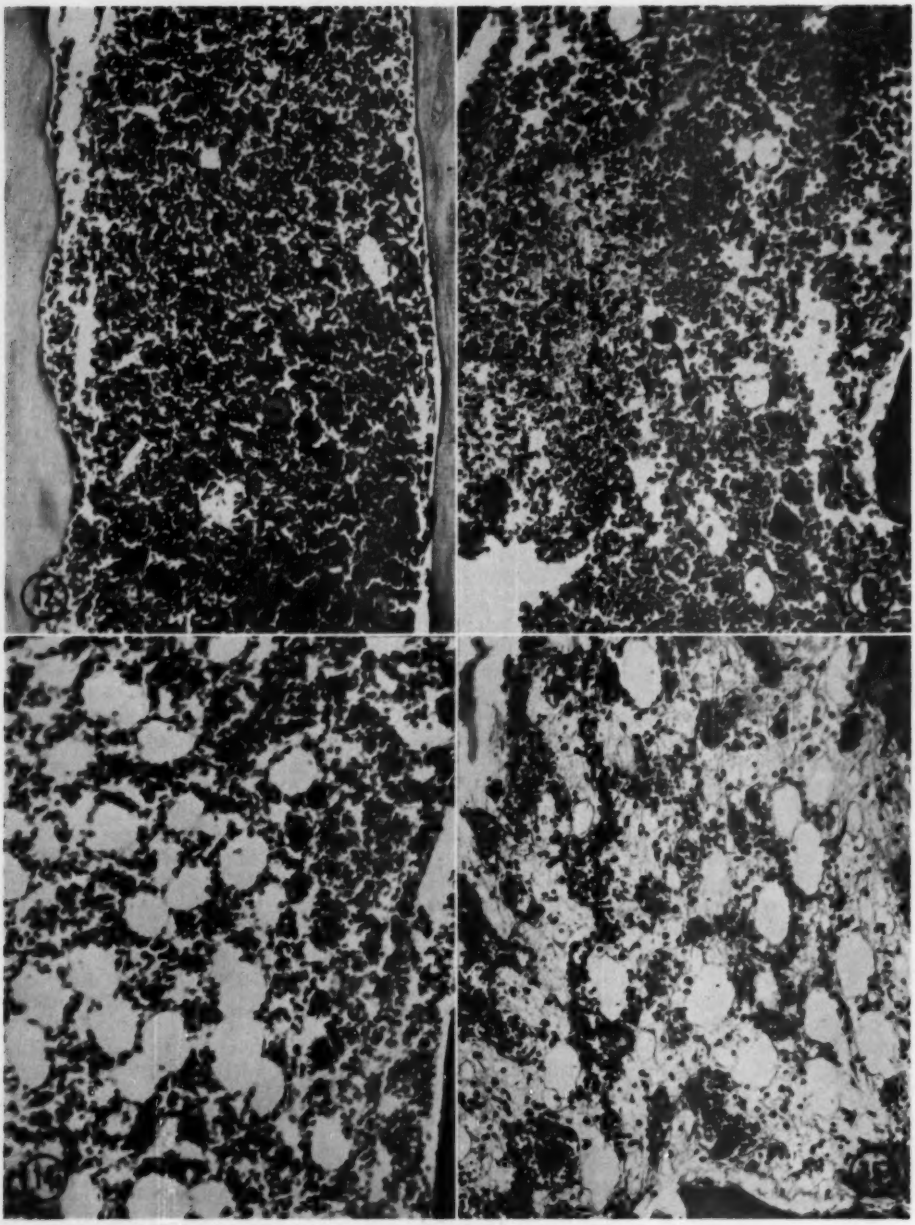

Fig. 12—Bone marrow, one hour after irradiation: highly cellular rib marrow. x 145. AFIP 248129.

Fig. 13—Bone marrow, twenty-nine hours after irradiation: reduction in erythroid and myeloid elements in rib marrow. Persistence of erythrocytes and megakaryocytes. x 145. AFIP 248135.

Fig. 14—Bone marrow, forty-five hours after irradiation: further decrease in rib marrow cellular elements; megakaryocytes still persisting. x 145. AFIP 248137.

Fig. 15—Bone marrow, 140 hours (six days) after irradiation: marked reduction in cellularity of rib marrow. Fibrinoid material in sinuses. x 141. AFIP 248145.

Collagen.—Changes in collagen were confined to swelling of the perivascular collagen, particularly in the lymph node sinuses. This is thought to be due to edema.

Urinary System.—The only change noted was the clumping of the erythrocytes in the glomerular capillaries, mentioned previously.

Respiratory System.—Clumping of erythrocytes as seen in the glomerular capillaries was also observed in the alveolar capillaries. There were no other perceptible changes.

Liver, Gall Bladder, Pancreas, Thyroids, Adrenals.—No changes were demonstrable with conventional methods of preparation.

Central Nervous System.—No demonstrable changes were observed using conventional methods of preparation.

Cardiovascular System.—No demonstrable changes were observed.

Musculoskeletal System.—Changes in bone and muscle could not be demonstrated throughout the entire series.

Discussion

Highly significant microscopic changes occurred in this series of animals in spite of the minimal gross changes. Gross multiple hemorrhages were observed only in the last dog of the series at 164 hours, on the seventh day after irradiation. Had the series been continued, the terminal phase of the irradiation syndrome with its hemorrhages, necrosis, cellulitis, and ulceration would have been expected. As it is, no ulceration or cellulitis was observed. The microscopic changes that pave the way for these terminal features were present in these animals. The depletion of the bone marrow with its concomitant agranulocytosis, for example, is the precursor of the typical necrosis and ulceration which are unaccompanied by any cellular reaction.

The large number of erythrocytes in the medullary sinuses of the lymph nodes and the associated erythrophagocytosis observed as early as one hour after irradiation are considered to be of special significance. It is a well-established fact[15,16] that some erythrocytes may normally be found in the medullary sinuses of lymph nodes. These erythrocytes may return to the peripheral circulation or eventually be phagocytized. It is generally believed that immediate phagocytosis of undamaged erythrocytes in the lymph node is unlikely. Work on amphibian larvae has substantiated this belief.[17] It has been shown that extruded undamaged erythrocytes remain in amphibian tissues for about fifteen hours before wandering phagocytic cells will engulf them.

It is thought that there may be injury to certain mature erythrocytes and to the capillary endothelium resulting in an increased capillary permeability. These erythrocytes then escape through the capillary walls and are conducted by the lymphatics to the lymph node sinuses where they are promptly phagocytized. The peculiar clumping of erythrocytes again suggests damage to the erythrocytes and the capillary endothelium.

It is believed that changes in the lymphatic tissues and hematopoietic tissues are consistent and regular enough to be used as indexes of radiation injury due to total body ionizing radiation. The changes in the lymphatic tissues are easier to trace, since the problem of identifying numerous types of cells encountered in the bone marrow does not arise. The difficulties of identification are greatly enhanced by morphological changes due to radiation injury and the decalcification and processing of the sections.

Summary

Eighteen dogs of the Walker Foxhound variety were exposed to 600 r. of bilateral total body x-radiation. These dogs were serially destroyed, autopsied, and the tissues studied grossly and microscopically. The essential histopathological changes were principally:

1) immediate and progressive degeneration of the lymphoid tissues with a transient repopulation of follicles;

2) early and progressive degeneration of the myeloid and erythroid elements of the bone marrow, progressing to complete aplasia with no evidence of regeneration during the period of observation;

3) evidence of early erythrocyte and capillary endothelial damage, as suggested by the early massive erythrophagocytosis observed in the lymph nodes, and the formation of aggregates of erythrocytes sug-

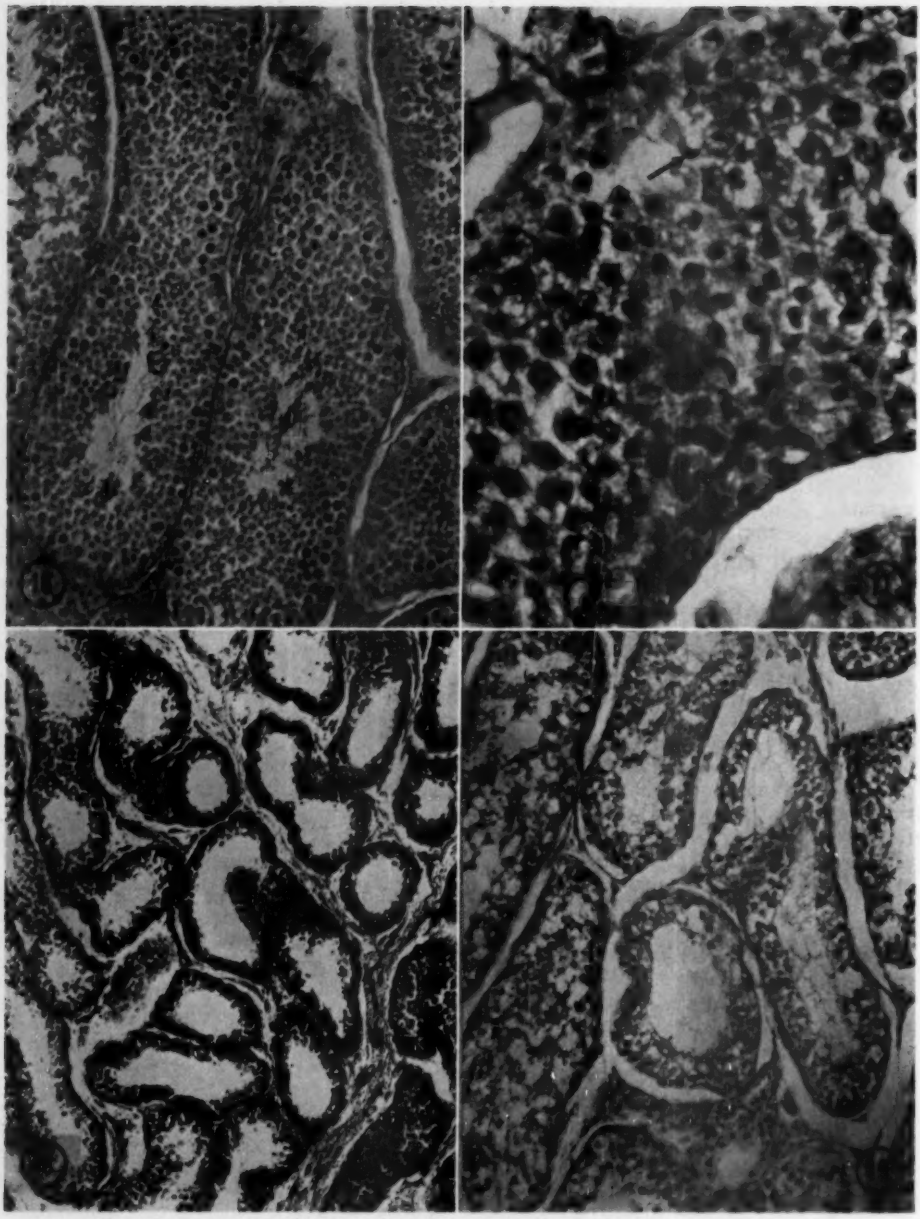

Fig. 16—Testis, one hour after irradiation: active spermatogenesis, numerous spermatozoa in tubules. x 135. AFIP 248129.

Fig. 17—Testis, twenty-nine hours after irradiation: nuclear changes in germ cells. Numerous crescents of nuclear material. x 430. AFIP 248135.

Fig. 18—Testis, sixty-eight hours after irradiation: marked decrease in spermatogenesis, aspermia. x 75. AFIP 248139.

Fig. 19—Testis, eighty hours after irradiation: marked reduction in spermatogenesis, peculiar vacuolization in tubules. x 105. AFIP 248140.

gestive of agglutinative thrombi within capillaries;*

4) early and progressive degenerative changes within the epithelial elements of the intestinal tract, with subsequent regeneration;

5) progressive patchy degeneration within the testicular tubules, resulting in decreased spermatogenesis and aspermia;

6) the development on the seventh day of the period of study of a hemorrhagic syndrome of indefinitely determined etiology.

References

[1] Wolbach, S. B.: The Pathological Histology of Chronic X-Ray Dermatitis and Early X-Ray Carcinoma. J. M. Res., *21*, (1909): 415-449.

[2] Warren, S.: Effects of Radiation on Normal Tissue. Arch Path., *34*, (1942): 443-450; 562-608; 749-787; 917-931; 1070-1084.

Ibid., *35*, (1943): 121-139; 353.

[3] Warren, S.: Histopathology of Radiation Lesions. Physiol. Rev., *24*, (1944): 225-238.

[4] Henshaw, P. S.: Experimental Roentgen Injury. I-IV. J. Nat. Cancer Inst., *4*, (1944): 477-522.

[5] Bloom, W.: Histopathology of Irradiation from External and Internal Sources, 1st ed., McGraw-Hill Book Co., New York, 1948.

[6] Barrow, J., and Tullis, J. L.: The Sequence of Cellular Response to Injury in Mice Exposed to 1100 r. Total Body X-Radiation. Naval Medical Research Institute Project NM-007039, Report No. 23, July 6, 1949, National Naval Medical Center, Bethesda, Md.

[7] Lamson, B. G., and Tullis, J. L.: The Progression of Morphologic Lesions in Swiss Mice Exposed to 625 r., 2,000 kv.p., Total Body X-Radiation. The Military Surgeon, *109*, (1951): 281-293.

[8] Tullis, J. L.: The Sequence of Pathologic Changes in Swine Exposed to the $LD_{100/30}$ of Total Body Super Voltage X-Radiation. The Military Surgeon, *109*, (1951): 271-280.

[9] Tullis, J. L., and Warren, S.: Gross Autopsy Observations in the Animals Exposed at Bikini. Preliminary Report. J. Am. M. A., *134*, (1947): 1155-1158.

[10] Tullis, J. L.: The Response of Tissue to Total Body Irradiation. Am. J. Path., *25*, (1949): 829-851.

[11] Bloom, W.: Plutonium Project: Histologic Changes Following Radiation Exposures. Radiology, *49*, (1947): 344-347.

[12] Warren, S.: The Pathologic Effects of an Instantaneous Dose of Radiation. Cancer Res., *6*, (1946): 449-453.

[13] Chambers, F. W., Jr., Morgan, J. E., and Istock, J. T.: Output Characteristics of a Commercial X-Ray Generator at 2,000 kv.p. Naval Medical Research Institute Project NM 006 012.08.26, Dec. 7, 1949, National Naval Medical Center, Bethesda, Md.

[14] Tullis, J. L., Chambers, F. W., Jr., Morgan, J. E., and Zeller, J. H.: Mortality in Swine and Dose Distribution Studies in Phantoms Exposed to Super Voltage X-Radiation. Naval Medical Research Institute Project NM 006 012.04.32, Aug. 29, 1950, National Naval Medical Center, Bethesda, Md.

[15] Maximow and Bloom: Textbook of Histology, 4th ed. W. B. Saunders Co., Philadelphia, Pa., 1943.

[16] Trautmann, A., and Fiebiger, J.: Lehrbuch der Histologie und vergleichenden mikroskopischen Anatomie der Haustiere, Paul Parey, Berlin, 1949.

[17] Clark, E. R., and Clark, E. L.: Intravascular Phagocytosis of Erythrocytes. Am. J. Anat., *41*, (1928): 227-248.

[18] Kahn, J. B., Jr., and Furth, J.: The Pathogenesis of Post-Irradiation Anemia. Blood, *7*, (1952): 404-416.

[19] Ross, M. H., Furth, J., and Bigelow, R. R.: Capillary Fragility Caused by Ionizing Radiations-Changes in Cellular Composition of the Lymph. Blood, *7*, (1952): 417-428.

* Since the completion of this work, two reports[18, 19] by Dr. Jacob Furth and his co-workers have appeared from the Oak Ridge National Laboratory presenting experimental evidence of erythrocyte damage and capillary fragility caused by ionizing radiation. Dr. Furth indicates that he believes increased capillary fragility to be an important factor in the hemorrhagic syndrome of ionizing radiation. Papers based on these reports have appeared in abbreviated form in the April, 1952, number of the journal *Blood*.

Actinomycosis (Nocardiosis) in a Dog with a Brief Review of this Disease

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SEVERAL CASES designated as actinomycosis have been reported in dogs, but many of them have been diagnosed without extensive bacteriological studies. Where bacteriological work was done, it was found that the isolated microörganisms varied. Some were aërobes and some were anaërobes.

There has been considerable confusion in the classification and nomenclature of the actinomycetes. The following names have been used by various authors for strains isolated from dogs: *Actinomyces baudeti*,[9] *Actinomyces bicolor*,[40] *Actinomyces canis*,[25] *Actinomyces israeli* var.,[24] *Cladothrix canis*,[32] *Cohnistreptothrix canis*,[5] *Discomyces pleuriticus*,[41] *Discomyces pleuriticus canis familiaris*, *Pleuromyces canis familiaris*,[38] *Nocardia bicolor*,[17] *Nocardia canis*,[10] *Oospora canis*,[35] and *Streptothrix canis*.[31] Balozet and Pernot[5] and Ginsberg and Little[19] have isolated an Actinomyces which they considered identical with Eppinger's *Cladothrix asteroides*.[13]

The microörganisms described would now probably be classified within the family Actinomycetaceae Buchanan[42] and are named accordingly.

Actinomycosis may occur in dogs at various sites.

Cutaneous and subcutaneous forms of the disease are commonly seen.[4, 5, 9, 21, 25, 30, 32, 38] Cutaneously, tumor-like growths may be seen. Subcutaneously, the infection develops granulomatous tissue which undergoes puriform transformation, and pus-filled fistulas are subsequently formed. The infection frequently affects the extremities but the neck and the abdomen may be involved. Study of the regional lymph nodes reveals a marked lymphadenitis, but suppuration is seldom seen.

Simultaneous with the cutaneous and subcutaneous lesions, there may be actinomycotic infection in the pleural or peritoneal cavities.

Thoracic actinomycosis has been reported in a number of cases.[2, 11, 14, 21–23, 29, 27, 33, 36, 37, 39, 40, 44] Bronchopneumonia, pulmonary abscesses, granulomatous lesions on the pleura and pleuritis with exudate have been found. In the latter instance, rapidly precipitating "sulphur granules," which were used in diagnosing the disease, were found. Several authors agree that these sulphur granules are not typical rosette formations as in bovine actinomycosis but mycelial masses without club formation. The bronchial lymph nodes may be involved.

Abdominal actinomycosis[1, 6, 8, 14–16, 20, 27–29, 34] may involve the peritoneum and the abdominal viscera, especially the liver, producing the clinical picture of chronic peritonitis with debilitation. On necropsy, varying degrees of peritonitis with granulomatous tissue are found. As in thoracic actinomycosis, groups of mycelial filaments (sulphur granules) precipitate rapidly from the exudate.

To our knowledge, only 1 case of oral actinomycosis has been reported. The symptoms were ulceration of the lips, gingivitis, foul breath, anorexia, edema of the lower jaw, and abscess formation of one of the submaxillary lymph nodes.[19] Filaments in the pus from the affected lymph node were gram-positive and acid-fast.

Another case which was not definitely diagnosed as, but was thought to be, oral actinomycosis presented numerous growths on the mucous membranes of the mouth.[26] Only the mandible has been reported as a site of osseous actinomycosis.[18, 42]

Two cases of actinomycosis affecting the nervous system have been described.[3, 43] In 1 case there were symptoms of respiratory embarrassment and convulsions, and at necropsy acute encephalitis with multiple foci, lymphadenitis of the bronchial lymph nodes, and nephritis were found. Evenly stained, acid-fast mycelia were evident in the lesions. One case of meningo-encephalitis presented small masses of actinomycotic filaments in a purulent fluid found in the ventricles. There were no other visible lesions.

The prognosis has nearly always been guarded in the reported cases of actinomycosis. A thorough surgical debridement with local application of iodine has been effective in a certain number of cases of cutaneous and subcutaneous actinomycosis.

Case Report

SYMPTOMS

The patient was a 7-month-old German Shepherd, reported ill for two days before admission. There were symptoms suggestive of distemper, such as vomiting, diar-

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The writers are indebted to Dr. R. Fagan, School of Public Health, Harvard University, for making the rabbit and guinea pig inoculations.

rhea, anorexia, and a constant rise in temperature. No respiratory symptoms were evident. The foot pads, normal on entry, became hyperkeratinized. After twenty days of hospitalization, the animal died.

NECROPSY

External examination revealed a thin, dehydrated animal with extremely thick, hard pads. Enlargement of the superficial lymph nodes, especially the right axillary and left prescapular, was noted. No fluid was present in the thoracic cavity. The right bronchial lymph node measured 3 by 2 by 2 cm. and fluctuated on palpation. When incised, the node was found to contain an abscess with pus that was yellowish grey, pasty, and odorless. Pressure from this lymph node on the right bronchus partially occluded the lumen, and the distended wall of the bronchus was very thin (fig. 1). The remaining surface of the node adhered to the right lung, and in the lung tissue adjacent to the lymph node there was consolidation 1 to 1.5 cm. in depth and the tissue was greyish white. Underlying this consolidated band, there was a narrow layer of atelectatic lung tissue. Congestion was present in the left lung. An abscess, which measured 0.5 by 1 by 1 cm., was found in the left bronchial lymph node. Sternal lymph nodes were enlarged but not abscessed.

Both kidneys were hydronephrotic. A narrow whitish grey area in the left kidney extended perpendicularly from the surface of the kidney through the cortex to the periphery of the medulla. This process was softer than surrounding renal tissue but it did not appear to be purulent.

Between the kidneys and surrounding the ureters there was a mass which measured 3 cm. in diameter (fig. 2) and contained an abscess with pus of the same character as that found in the pulmonary lymph nodes. This mass constricted the lumina of the ureters and involved the renal lymph nodes.

A yellowish grey, nodular process was present in the mesentery adjacent to the small intestine. It measured 1.5 by 1 by 1 cm. and its contents were of the same character as those of the processes previously described. Fibrinous adhesions were found

Fig. 1—Dorsal view of the lungs with incised trachea, bronchi, and right bronchial lymph node. The abscessed lymph node partially occludes the underlying right bronchus. x 2.

between this mass and a loop of the small intestine, but only the serosa of the intestine was involved. The mesenteric lymph nodes were enlarged but did not show any abscess formation.

HISTOPATHOLOGY

Right Bronchial Lymph Node.—The nonabscessed part of the node was divided by bands of edematous connective tissue into areas containing numerous polymorphonuclear cells. In many areas there was a zone of large mononuclear cells with vesiculate nuclei outside the accumulation of polymorphonuclear cells, partially separating these cells from the fibrous bands. A few multinucleated giant cells were present among the mononuclear cells. Foci of fragmented pyknotic cell nuclei in acidophilic material were disseminated in the areas of polymorphonuclear cell infiltration. Mildly acid-fast rods and filaments were evenly distributed throughout the areas in which the polymorphonuclear cells were present. Many of the rods and filaments contained gram-positive granules.

Right Lung.—The consolidated pulmonary tissue adjacent to the lymph node contained areas in which the alveolar pattern was intact, and the alveoli contained polymorphonuclear and large mononuclear cells. Other areas were more heavily infiltrated with polymorphonuclear cells and the alveolar pattern could not be distinguished. A few mononuclear cells were found scattered among the polymorphonuclear cells. Ziehl-Neelsen stained sections revealed short, mildly acid-fast, unevenly stained filaments which contained gram-positive granules. Regions not actively involved in the granulomatous process showed small areas in which the alveoli contained many large mononuclear cells and a few giant cells and their walls were thickened by swelling and proliferation of the alveolar cells. The cytoplasm of these mononuclear cells was vacuolated and some of them contained acidophilic cytoplasmic inclusion bodies. Sudanophilic droplets were demonstrated in frozen sections. These changes were characteristic of those seen in the interstitial pneumonia that may accompany canine distemper.

Fig. 2—Incised right kidney. There is dilatation of the pelvis. A spherical process surrounds the ureter.

Left Kidney.—The elongated process extending through the cortex contained polymorphonuclear cells and was separated from the surrounding renal tissue by a peripheral layer of large mononuclear cells. There was only a rather slight fragmentation of the nuclei in the polymorphonuclear cells, indicating that tissue necrosis was limited. Scattered rods and filaments measuring 30 to 40 μ in length were present. They were nonacid-fast and contained gram-positive granules. Small foci of predominantly mononuclear cells were present throughout the cortex.

Adrenals.—An isolated nodule was found in the cortex of one adrenal. An area of cellular debris and nuclear fragments in the center of the nodule was surrounded by a broad zone of large mononuclear cells. This process displaced the cortical tissue. There was no inflammatory reaction in the tissue surrounding the process. Ziehl-Neelsen stains did not show acid-fast microorganisms; however, rods and filaments containing gram-positive granules were present.

Mesenteric Lymph Node.—There was proliferation of the reticular cells, many of which contained large, slightly basophilic intranuclear inclusion bodies. Plasma cells were scattered throughout the node. Microorganisms could not be demonstrated.

Lesion Between the Kidneys.—This was similar to that found in the right bronchial lymph node.

Right Axillary Lymph Node.—This node contained an extensive inflammatory process consisting of polymorphonuclear cells and cellular fragments lying in an acidophilic substance. Many nonacid-fast rods and filaments containing gram-positive granules were present.

Urinary Bladder.—Several of the epithelial cells contained cytoplasmic acidophilic inclusion bodies. Sections from the stomach, intestines, other abdominal organs, and the heart were found normal.

Summary.—The histopathological examination showed lesions of both canine distemper and a metastasizing granulomatous infection. The size and location of the latter lesions suggest that the processes in the lung and bronchial lymph nodes were the original site of infection. The age of the lesions could not be estimated. Death probably was due to respiratory embarrassment and malnutrition caused primarily by distemper and secondarily by the granulomatous infection.

Bacteriology

Material taken aseptically from the right bronchial lymph node was used for the bacteriological examination. Inoculation was made on chocolate agar under aërobic, micro-aërophilic and anaërobic conditions at 37 C. for primary isolation. Under aërobic and micro-aërophilic conditions, minute colonies were seen after forty-eight hours of incubation, becoming plainly visible at seventy-two hours. They were flat, greyish white, with a small porcelain-white elevated center, and were firmly attached to the culture medium. The largest colonies were less than 0.5 mm. in diameter. When touched with a platinum wire, the colonies lost

Fig. 3—Colonies on a blood agar plate after two weeks' incubation at 37 C. The surfaces are finely granular and the edges are fimbriated. x 20.

their chalky appearance and became grey. All the colonies had the same appearance. Smears made from the growth showed rods and filaments identical with those seen at the microscopic examination of the contents of the lymph node. There was no anaërobic growth, and subcultures from aërobic growth did not develop under anaërobic conditions. The presence of acid-fast rods and filaments in smears from the cultures supported the supposition that the infection was actinomycosis. Since the microörganisms grew under aërobic or microaërophilic conditions, but not under anaërobic, it was considered to belong to the genus Nocardia. The term "Nocardia Trevisan" for aërobic forms and the term "Actinomyces Harz" for anaërobic or micro-aërophilic forms of the family Actinomycetaceae Buchanan were suggested by Waksman and Henrici.[46]

Subcultures on mediums suitable for growth and differentiation of Nocardia were undertaken. Strains of Nocardia vary in staining and growth characteristics, pigment formation, hemolysis, pathogenicity, proteolytic activity, and certain other biochemical properties. Quadruple sets of mediums were used for subcultures. All utensils, culture plates, and tubes were sealed with scotch tape after inoculation to make possible long incubation without excessive evaporation. Cultures were incubated at 37 C. and at room temperature (25 C.) for at least eight weeks (fig. 3).

On most mediums at 37 C. growth was evident after thirty-six to forty-eight hours. At room temperature, the initial growth was slower. At the end of ninety-six hours, the colonies at both temperatures were identical and continued to show the same characteristics for several days. Later, certain differences, for examples in pigment formation, became apparent. Repeated culturings showed unchanged growth rate, and gross cultural features remained constant.

The culture characteristics are summarized in table 1.

In gelatin, chopped meat, brom-cresol-purple milk, plain milk, nitrate broth, and bactotryptone solution, surface growth gradually changed from white to yellowish red, especially at room temperature.

Hemolysis.—An unusual type of hemolysis was noted on blood agar plates incubated at room temperature. After six weeks' incubation, the bright red medium suddenly became paler, and turned brownish red except for a round, well-defined zone 6 to 10 mm. in diameter surrounding the colonies. Just inside the periphery of this red zone, the medium was clearer and by the following day a halo of hemolysis 0.5 mm. in width had developed. The original periphery remained as a dark, narrow ring which was separated from the nonhemolysed central area by the zone of hemolysis. On the third day, there was clearing with formation of another ring of hemolysis in the peripheral part of the dark central zone. This phenomenon was fairly distinct for four or five days. After this time, the nonhemolysed rings gradually became paler, as did the remaining central area. On prolonged incuba-

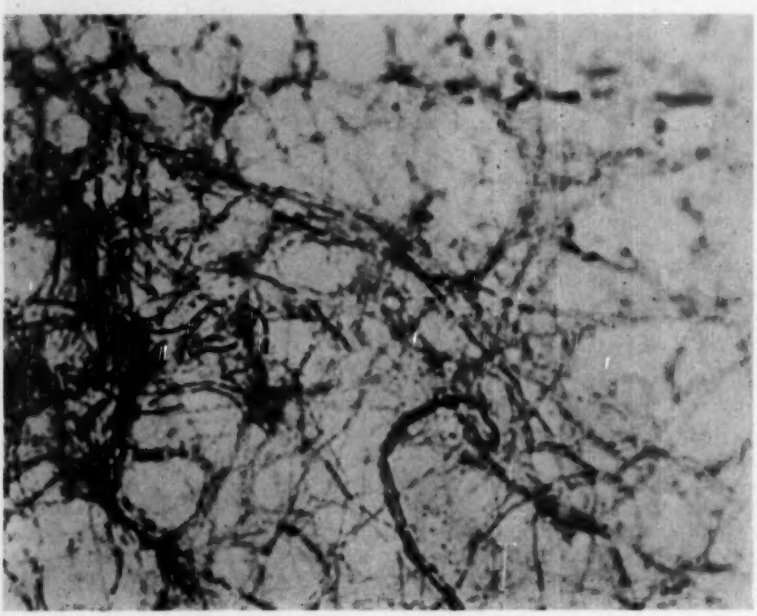

Fig. 4—Ziehl-Neelsen stained smear of material from colony.

The dark elements are the acid-fast rods and filaments. Many nonacid-fast elements are present. x 1750.

tion, the ring formation disappeared and after ten or twelve days there was complete hemolysis.

The strain showed lack of visible proteolytic activity in Loeffler's serum, gelatin, and chopped meat and did not show activity in tryptone solution, starch-serum-water, and brom-cresol-purple milk. Nitrites were produced from nitrates.

In arabinose, dextrose, dulcite, lactose, maltose, mannitol, rhamnose, sorbitol, sucrose, xylose, and salicin, no acid or gas was formed.

Phenol Medium.—There was no growth in nutrient broth containing 0.5 per cent phenol at either temperature.

Odor.—Cultures in all mediums had a distinctly mouldy smell which resembled that of garden soil.

Smears From Pus and Cultures.—Four stains were used: methylene blue, Gram's stain, Ziehl-Neelsen's, with decolorization in 25 per cent sulphuric acid or 3 per cent hydrochloric acid in 95 per cent alcohol, and Abbot's spore stain. The growth was compact and it was difficult to make smears except by rubbing the material between two slides. No significant differences were found in smears from pus and solid or fluid mediums. Both acid-fast and nonacid-fast rods and filamentous rods and coccoid elements were present in the pus as well as in the cultures. The rods measured 0.50 to 0.75 μ in diameter. Accumulations of rods and filaments but no sulphur granules were observed in the pus. There was true branching of a few of these filaments, some of which ranged between 50 and 75 μ in length. Methylene blue did not stain the filaments uniformly; unstained spaces remained between blue segments. In many filaments,

TABLE 1—Culture Characteristics of the Microörganism at 25 and 37 C.

Medium	Colony-form	Color	Remarks
Plain, blood and chocolate agar	Flat	Greyish white; central chalky elevation; uniformly white and farinous after a few days.	After 2 or 3 weeks, the growth showed pitting with surface depression about 0.25 mm. in diameter. A peculiar phenomenon of hemolysis on blood plates at 25 C. was noted.
Sabouraud's maltose agar	Flat in the beginning; conical or hemispherical after 6 to 8 days.	Greyish yellow on the surface; yellow woolly subsurface growth which became yellowish brown; no diffusion of pigment.	Craters 0.25 mm. in diameter appeared in the surface. Later a minute white spot developed in the center of the large craters.
Nutrient broth	Dome-shaped colonies on the surface; round colonies at the bottom.	After long incubation the surface of the floating colonies attained a pale pinkish cast.	
Fluid horse serum	Flat confluent surface colonies with a shallow depression in the center; spherical colonies at the bottom.	Yellowish grey surface growth. Bottom colonies white with dark yellow centers.	
Petragnani	At 37 C.—Convex colonies after 48 hrs. More elevated on 4th day.	White at 37 C.	At 37 C.—After 2 or 3 weeks small distinct yellow areas appeared in the white superficial layer of confluent growth. At the same time the surrounding surface started to lose its chalkiness. After 4 weeks the entire surface was creamy yellow. It retained this color for 4 weeks, at the end of which the surface became white again.
	At 25 C.—Convex colonies visible after 3rd day.	Yellow at 25 C.	At 25 C.—Yellow color was retained after prolonged incubation and there was some similarity to colonies of *Mycobacterium tuberculosis*.
Glycerol agar (3 % glycerol)	Flat	White	Confluent growth was rugose with many small pores visible in a mealy superficial layer. The pores enlarged and appeared as bare spots. Dew drops appeared on the surface and dried leaving a brownish deposit.
Potato	Flat	White	
Carrot	Flat	White at 25 C. and 37 C.; later yellowish red at 25 C.	
Gelatin			At room temperature the growth resembled an inverted fir tree, the growth being most dense at the base.

there were round deep blue granules in the blue segments. A few filaments were two to four times the usual diameter and contained groups of small dark blue granules. Gram's stain, like methylene blue, colored the filaments variably. The stained portions of the filaments contained gram-positive granules. Ziehl-Neelsen stain revealed a few long, thin, uniformly acid-fast filaments that contained granules which stained blue. In most of the filaments, the acid-fastness was confined to short segments (1 to 2 μ) which were in close proximity (fig. 4). The acid-fast segments contained blue granules or particles, many of which protruded from the surface of the filaments. Unstained centers could be seen in many of the larger granules. Many nonacid-fast elements were present. The acid-fastness was very pronounced both in smears from pus and from cultures, since the elements resisted decolorizing for more than five minutes. The swellings in the filaments in the hanging-drop preparation (fig. 5) were mildly acid-fast with a nonacid-fast elongated space in the center.

With Abbott's spore staining of colony material, a few pale blue granules could be recognized in some of the treads.

Morphology and Reproduction.—The microörganism was studied on Sabouraud's agar medium on a hanging-drop slide at 37 C. and at room temperature. Surface growth from Sabouraud's agar was used as inoculum. The cover glass was sealed with paraffin on three edges. The cultures were kept in a moist atmosphere and examined daily under the microscope. Immediately after planting, large numbers of coccoid elements, rods, and a few filaments were seen among clumps of inoculums. Many of the rods were club-shaped. There were long filaments in the clumps. Growth had begun after twenty-fours hours' incubation at 37 C. It was manifested by filamentous projections on the coccoid forms and elongation of the rod forms. The longest filaments measured about 50 μ. On the day following planting, filaments radiated from growth centers and secondary branches, measuring as much as 25 μ, projected from them; the longest filaments were about 75 to 100 μ. On the third day, the growth centers appeared as dense accumulations of filaments.

The filaments, very slender (0.5 to 0.75 μ), wavy, and greenish yellow, contained granules. The granules were black in reduced light and re-

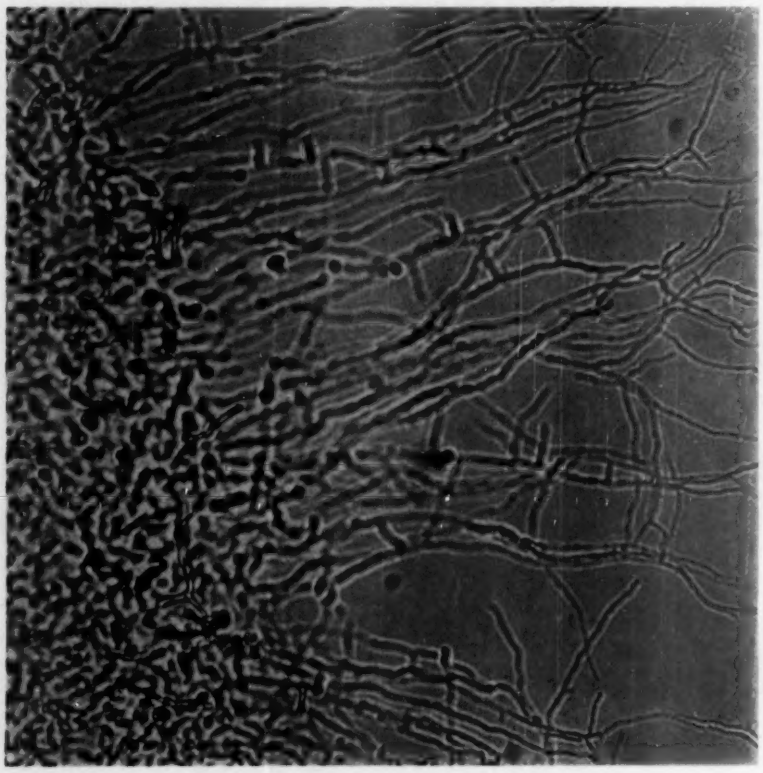

Fig. 5—Growth in hanging-drop slide after four weeks' incubation. Fragmentation has taken place in the mycelial mass at the left. Long granule-containing filaments are seen at the right. x 1750.

fractive in intense light. Young filaments were uniform in thickness. Later, swellings (6 to 7 μ in diameter) developed on some of the filaments giving them a moniliform appearance. After about four weeks, the granules became more numerous and distinct in some of the filaments, and these filaments fragmented into rods of varying lengths (fig. 5). In some instances, the newly formed rods lay in the same position as in the filaments; in other instances, they lay in random polarity. This phenomenon of disorganization appeared rather suddenly and on succeeding days more and more filaments dissociated.

In many of the filaments, the extremities were at first straight but later became curved. In some cases, loops were formed by the curled ends, giving the appearance of sporelike bodies.

At room temperature, there was no growth until after three or four days' incubation. The growth was slower and the fragmentation occurred later than at 37 C.

Pathogenicity.—Guinea pigs were inoculated intraperitoneally and rabbits intraperitoneally and intravenously. Each animal was given 4 to 5 colonies from 8-day-old cultures. None of them developed lesions.

Resistance.—Suspended in physiological saline solution, the microörganism resisted a temperature of 60 C. for thirty minutes but not for longer periods of time.

Susceptibility of the Strain to Antibiotics In Vitro.—Susceptibility to penicillin, streptomycin, and aureomycin was determined on blood plates by the diffusion method. The thickness of the medium was 2 mm. and the holes were 7 mm. in diameter. Aqueous solutions of crystalline potassium penicillin G, dihydrostreptomycin sulphate, and aureomycin hydrochloride with sodium glycine buffer were used and the readings were taken after five days' incubation at 37 C. The lowest concentrations of the three antibiotics that gave total inhibition of growth in a zone around the hole were: penicillin—30 I.U./cc., streptomycin—0.05 mg./cc., aureomycin*—0.6 mg./cc. The results could be reproduced.

Discussion

From the nature of the lesions and the primary microscopic examination in which a gram-positive, acid-fast, pleomorphic microörganism was found, it was assumed that the infection was actinomycosis.

The microörganism was easily cultivated on different mediums under aërobic and micro-aërophilic conditions with a more rapid initial growth at 37 C. than at room temperature. It was acid-fast in primary cultures and retained its acid-fastness on subculturing; however, it was nonacid-fast or very slightly acid-fast in the tissue. Microscopic and cultural characteristics differentiated the organism from *Mycobacterium tuberculosis.*

The evidence derived from the reproduction, the growth, and the physiological characteristics related the strain isolated to *Actinomyces asteroides* or *Nocardia asteroides* as it is now classified in Bergey's manual.[7]

In other respects, the strain differed from *N. asteroides.* The latter does not grow in gelatin along the stab, while this strain produced an inverted fir treelike growth along the stab at room temperature. There were also other differences, especially in pigment formation. *Nocardia asteroides* on plain agar becomes yellow or yellowish red, and on potato is white at first, then yellow, and at last brick red. The strain showed some similarity to *Nocardia polychromogenes*[7] which also grows along the stab in gelatin. Since most of the strains isolated from lesions in dogs denoted as actinomycotic have been incompletely described, correlation is difficult.

Acid-fast strains of Actinomyces have been described in dogs by Rabe,[32] Trolldenier,[40] Ginsberg and Little,[19] and other authors. Ginsberg and Little considered one of their strains similar to *A. asteroides.*

The strain isolated by the authors does not appear to be identical with any of these described, and it is also different from *Actinomyces pyogenes,*[12] *Actinomyces minimus,*[12] and *Actinomyces farcinicus*[12] which may be pathogenic to dogs.

The term "actinomycosis" has been employed in the literature to denote this type of infection; however, according to the modern nomenclature, this case was diagnosed as nocardiosis.

Summary

1) Literature concerning the various forms of canine actinomycosis is reviewed.

2) Clinical and pathological findings of a case of canine distemper (hard pad disease) complicated by actinomycotic lesions in the lungs, kidneys, and other abdominal organs are presented.

3) Bacteriological study of the microorganism indicates that it is of the genus Nocardia as now classified in Bergey's Manual.

* The effect of aureomycin is inhibited by peptone. The basis for the blood agar plates in the experiment was tryptic-digested broth without peptone added.

References

[1] Bader, D. N.: Canine Actinomycosis. North Am. Vet., *30*, (1949): 781-782.

[2] Bahr, L.: Ueber Actinomycose beim Hunde. Ztschr. f. Tiermed., *8*, (1904): 47-67.

[3] Balozet, L., and Pernot, P.: Meningite du Chien Causée par un Actinomyces. Bull. Acad. Vét. France, *9*, (1936): 168-177.

[4] Barke: cited by Curasson, G.: Maladies infectieuses des Animaux Domestiques. Vigot Frères, Paris, *2*, (1947): 434.

[5] Baudet, E. A. R. F.: L'Actinomycose du Chien. Ann. Parasitol. Hum. Comp., *12*, (1934): 296-308.

[6] Baumgaertner, H.: Beitrag zur Aktinomykose der Kleintiere. Tierärztl. Rundschau., *36*, (1930): 585-587.

[7] Bergey's Manual of Determinative Bacteriology. 6th ed. Williams & Wilkins Co., Baltimore, 1948.

[8] Brion, A.: L'Actinomycose du Chien et du Chat. Rev. Méd. Vét., *91*, (1939): 121-159.

[9] Brion, A.: L'Actinomycose du Chien et du Chat. Formes Cliniques. Necrobacillose des Carnivores. Pathogenie, Pluralité des Espèces d'Actinomyces. *Actinomyces baudeti*. Rev. Méd. Vét., *93*, (1942): 145-157.

[10] Chalmers, A. J., and Christopherson, J. B.: A Sudanese Actinomycosis. Ann. Trop. Med., *10*, (1916): 223-282.

[11] Curasson, G.: Maladies Infectieuses des Animaux Domestiques. Vigot Frères, Paris, *2*, (1947): 433-434.

[12] Dodge, C. W.: Medical Mycology. C. V. Mosby Co., St. Louis, (1935): 694-785.

[13] Eppinger, H.: Ueber eine Neue Pathogene Cladothrix und eine Durch die Hervorgerufene Pseudotuberculosis (Cladothrichica). Beitr. Path. Anat. u. z. allg. Path., *9*, (1891): 287-328.

[14] Eroms, H.: Aktinomykose beim Hund. Deutsche tierärztl. Wchnschr., *47*, (1939): 437-438.

[15] Feldman, W. H., and Mann, F. C.: Lesions in the Stomach of a Dog. Simulating Actinomycosis. J.A.V.M.A., *81*, (1932): 83-91.

[16] Forsyth, H.: Actinomycosis in a Labrador Bitch. Vet. Rec., *61*, (1949): 586.

[17] Froilando de Melle, I., and St. Antonio Fernandes, I. F.: cited by Dodge, C. W.: Medical Mycology. C. V. Mosby Co., St. Louis, (1935): 694-785.

[18] Famagalli, A.: Actinomicosi in un Cane. Clin. Vet., *26*, (1903): 208-209.

[19] Ginsberg, A., and Little, A. C. W.: Actinomycosis in Dogs. J. Path. Bact., *60*, (1948): 563-572.

[20] Gohn, H. W.: Actinomycosis in a Dog. J. Comp. Med. Vet. Arch., *23*, (1902): 241-242.

[21] Hartl, R.: Casuistische Beiträge zur Actinomycose bei Thieren. Berl. tierärztl. Wchnschr., *17*, (1901): 1-6.

[22] Hutyra, F., Marek, J., and Manninger, R.: Special Pathology and Therapeutics of the Diseases of Domestic Animals. 5th ed. Bailliere, Tindall, and Cox, London, (1949): 713-714.

[23] Kitt, Th.: Aktinomykotische Brustfellentzüdung beim Hunde. Monatsh. prakt. Tierh., *29*, (1919): 518-526.

[24] Lesbouyries, G.: Actinophytose à Actinomyces chez les Carnivores. Rec. Méd. Vét., *118*, (1942): 193-202.

[25] Levy, E.: Ueber die Actinomycesgruppe (Aktinomyceten) und die ihren. Verwandten Bakterien. Cbl. Bakt., *26*, (1899): 1-11.

[26] Madsen, D. E.: Some Studies of Three Pathogenic Fungi Isolated from Animals. Cornell Vet., *32*, (1942): 383-389.

[27] Martin, H. M.: Actinomycosis of the Dog and Cat. Vet. Exten. Quart., *87*, (1942): 15-19.

[28] Morant, K. M.: Actinomycosis in a Dog. Vet. Rec., *63*, (1951): 82.

[29] Michailov: cited by Brion, A.: L'Actinomycose du Chien et du Chat. Rev. Med. Vet., *91*, (1939): 121-159.

[30] Migliano, M. F., and Stopiglia, A. V., abstracted by Lopez-Pacheco, A. O.: Actinomycosis in a Dog. J.A.V.M.A., *118*, (1951): 51-52.

[31] Musgrave, W. E., and Clegg, M. T. (with bibliography by Polk, M.): Streptotrichosis with Special Reference to the Etiology and Classification of Mycetoma. Philippine J. Sci., *3*, (1908): 447-544.

[32] Rabe, C.: Ueber einen Neuentdeckten, Pathogenen Mikroorganismus bei dem Hunde. Berl. tierärztl. Wchnschr., *4*, (1888): 65-68, 77-78.

[33] Rivolta, S.: Del Micellio e della Varieta e Specie di Discomycete Patogeni. Giorn. Anat. Fisiol. Patol. Animali, Pisa, (1884): 190.

[34] Robin, V., Lesbouyries, G., and Vechiu, A.: Sur une Péritonite Mycosique des Carnivores. Bull. Soc. Cent. Méd. Vét., *79*, (1926): 332-336.

[35] Sartory, A.: cited by Brion, A.: L'Actinomycose du Chien et du Chat. Rev. Med. Vet., *91*, (1939): 121-159.

[36] Schlegel, M.: Bericht über die Tätigkeit des Tierhygienischen Instituts der Universität Freiburg i. Br. im Jahre 1910. XVI. Ztschr. f. Tiermed., *15*, (1911): 248-250.

[37] Schnelle, G. B.: *Streptotrichosis Canum*—Pseudoactinomycosis of a Dog. J.A.V.M.A., *74*, (1929): 789-790.

[38] Schroeder, H., and Hemmert-Halswick: Ueber eine Aktinomykoseartige Erkrankung der Haut beim Hunde. Tierärztl. Rundschau., *34*, (1928): 543-547.

[39] Torrance, F.: Actinomycosis in a Dog. J. Comp. Med. Vet. Arch., *21*, (1900): 421-523.

[40] Trolldenier: Ueber eine bei einem Hunde Gefundene Pathogene Streptothrix. Ztschr. f. Tiermed., *7*, (1903): 81-109.

[41] Vachetta, A.: Studi e Ricordi Clin. Milano (1882). Cited by Rivolta, s.: Del Micellio e della Varieta e Specie di Discomycete Patogeni. Giorn, Anat. Fisiol. Patol. Animali, Pisa, (1884): 190.

[42] Vachetta, A.: Osteocondrosarcoma Macrocellulare con Actinomyceti alla Mandibola Inferiore d'um Cane. Clin. Vet., *5*, (1882): 226-236.

[43] Waksman, S. A., and Henrici, A. T.: The Nomenclature and Classification of the Actinomycetes. J. Bact., *46*, (1943): 337-341.

[44] White, H. S.: Actinomycosis of the Lungs in a Dog. Cornell Vet., *35*, (1945): 72-74.

General References

Feldman, W. H.: Botryomycosis in a Dog. Cornell Vet., *22*, (1932): 50-55.

Henrici, A. T.: Henrici's Molds, Yeasts, and Actinomycetes, by Charles E. Skinner *et al.* 2nd ed. John Wiley & Sons, Inc., New York, (1948): 371-384.

Henrici, A. T., and Gardner, E. L.: The Acidfast Actinomycetes, with a Report of a Case from Which a New Species Was Isolated. J. Infect. Dis., *28*, (1921): 232-248.

Avianization of Lapinized Rinderpest Virus

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LITERATURE CITED

Strain 3 of lapinized rinderpest virus introduced by one of the authors and his co-workers[3-7] has been accepted by a number of authors in different countries as a suitable vaccine for cattle and buffaloes. Its applicability seems to cover bovine breeds of a relatively wide range of susceptibility. Satisfactory results of vaccination attempts were reported in native cattle and/or buffaloes by Isogai[8] in Mongolia, by Cheng and Fishman[9] in China, by Kuptabutra, Wongsongsorn, and Disayamaru[10] in Thailand, by Alton[11] at Hongkong, by Fulton[12] at the Gambia in West Africa and by Reisinger[13] in Formosa. Various breeds of cattle of European type were also successfully vaccinated with this strain by Kwong and Lee[14] in China, by Alton[11] and by Watson[15] at Hongkong.

Certain breeds of cattle, however, seem more highly susceptible to rinderpest than any of the animals mentioned above and can not withstand inoculation with this strain. According to Nakamura and Kuroda,[6] 29 per cent of Korean calves receiving the lapinized virus, during the passages from 100 to 360, died of rinderpest. From results of several experiments with Korean calves carried out by Nakamura,[16] up to the 550th passage and since then by Lee[17] up to date (over 1,000 passages), the strain has shown no distinctive evidence of progressive attenuation for this breed. A recent study by Fukusho[18] has shown that Japanese native cattle are as susceptible to rinderpest as Korean cattle. In each of their three experiments, in which the lapinized virus at the 839th passage of the original strain, the 849th and 860th passages of the China side strain were inoculated respectively into 6 Japanese calves, one-third of the animals died of rinderpest infection. When Fukusho and Reisinger[19] inoculated the lapinized virus of China-Formosa side strain, probably over 900 passages, into 6 Japanese and 3 Holstein-Friesian calves, all the Japanese calves displayed moderate to heavy infection and 2 died, while the Holstein-Friesians revealed only thermal reaction with or without illness and all survived.

The lapinized virus is still too pathogenic for these breeds of cattle and can not be used for the practical purpose of vaccination. At the same time, it is hardly expected that this strain may become more attenuated and safe for these hypersusceptible breeds by further rabbit passages. It was, however, thought that adaptation of the virus to a less closely related animal might result in further attenuation. From this viewpoint, serial passages of the virus through chicken embryos was considered a method worthy of trial.

During 1942 to 1945, Nakamura, Sato, and Miyamoto[20] studied the behavior of lapinized virus in the chicken embryo. They inoculated the virus into the vein in the chorioallantoic membrane of 12- to 14-day developing chicken embryos and traced the fate of the virus by means of rabbit titrations. They found that the virus so inoculated actually propagated in the embryo spleen, reaching its highest concentration in seventy-two hours. Attempts to transfer the virus to the next embryo spleen were, however, unsuccessful. The highest virus titer to be reached in the embryo spleen of the first generation was 12.5 rabbit m.i.d.* per milligram. This virus amount represented all that could be transferred to the next egg, as 0.05 cc. of 1 : 50 suspension, *i.e.*, 1 mg. of the tissue, was the largest dose inoculable to the egg through the intravenous route. This amount of virus in the inoculum was, however, definitely insufficient, as the inoculation of at least 100 rabbit m.i.d. was required for propagation of the virus in the chicken embryo spleen. After repeated failures in the embryo passage of lapinized virus, the same authors tried four lines of serial embryo passages of bovine virus. The same technique was employed. Twelve- to 14-day eggs were inoculated intravenously with 0.05 cc. of 1 : 50 virus suspension and then incubated at 38 C. for seventy-two hours. The embryo spleen was then harvested and transferred to the next egg at a dilution of 1 : 50 to 1 : 100. The results obtained from the inoculation tests in calves were encouraging. The first line was virulent after one, three, and five passages but not after ten; the second line was virulent after three but not after seven; the third line was virulent after four but not after seven; and the fourth line was virulent after four, six, eight, ten, and 14 but not after 20 passages. In the last line, apparent attenuation of the virus was noticed after ten passages, *viz.*: 1 of the 2 calves receiving the tenth passage virus died after a prolonged course of an eleven-day illness and 1 of the 2 calves receiving the fourteenth passage virus withstood the virus with mild symptoms and acquired solid immunity. But the study was then discontinued.

During almost the same years, attempts to attenuate bovine rinderpest virus under similar principle, *i.e.*, through chicken embryo passage, had

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* Minimum infectious dose.

been undertaken by the United States-Canadian scientists at the Grosse Isle laboratory [21-24] with successful results. These workers adapted the Kabete "O" strain to the chicken embryo by eight to 15 or more preliminary passages on the chorioallantoic membrane and then transmitted the virus in series in chicken embryos by way of the yolk sac. Extended transfers by the chorioallantoic route, as far as the ninetieth serial passage, maintained the virus in sufficient virulence to produce a fatal infection in calves of European type; on the other hand, transfer of the virus by way of the yolk sac resulted in a rather rapid attenuation of the virus for these animals. Attenuation of the virus to the point where it was suitable for vaccine purposes varied with each series of egg cultures. In one series, 19 yolk-sac passages provided sufficient attenuation while in another, 24 passages were required. In the opinion of the Grosse Isle workers, attenuation of the virus by means of repeated transfers in the yolk sac is a progressive process and it is reasonable to assume that after numerous such transfers the virus will no longer produce immunity in calves. Their experience on one occasion was that the virus which had been carried through 58 consecutive passages in the yolk sac failed to produce immunity in a calf, while the fifty-third passage from this series did produce immunity in several animals.

As to the adaptation of other bovine strains for chicken embryos, the Grosse Isle method did not always give similar results. Hale and Walker [24] touched on this subject in their paper from Grosse Isle. They failed to adapt an Indian strain to chicken embryos by yolk-sac inoculation after 30 preliminary chorioallantoic passages, at which point the series was discontinued. They could not transfer a North African strain even on chorioallantoic membranes. Closely following the Grosse Isle method, Cheng, Chow, and Fishman [25] made 12 unsuccessful attempts to adapt two Chinese and one Cairo strains to chicken embryos. In subsequent attempts with the Kabete strain, however, the same authors were successful in developing a yolk-sac series after 17 preliminary passages on chorioallantoic membranes. Daubney [26] was also successful in adapting the Kabete O strain to growth in the yolk sac after 71 chorioallantoic membrane passages.

Concerning the problem of whether the Grosse Isle method can be applied for the adaptation of goat or rabbit passage virus to growth in the egg, we refer to two experiments conducted at Grosse Isle. According to Hale and Walker,[24] a caprine strain, of which history and character were not given, was not adapted after 19 membrane passages but was capable of growth in the yolk sac after 37 such passages. Walker [27] was successful in adapting the Kabete virus which had been serially passaged through 5 rabbits to chicken embryos both on the chorioallantoic membrane and then in the yolk sac but was not successful with the same strain of virus which had been passaged through 65 rabbits.

In April, 1948, we were given the second opportunity to attempt the cultivation of lapinized rinderpest virus in chicken embryos and, if possible, by serial passages through chicken embryos, to attenuate it for cattle of the highest susceptibility, such as Japanese and Korean types. Work has advanced favorably and our aim has been nearly attained. This paper is a brief description of the work to date.

MATERIALS AND METHODS

Virus Source.—The lapinized rinderpest virus of strain 3, within a level of 733 to 740 rabbit passages, was used as the initial virus for cultures in chicken embryos.

Preincubation of Eggs.—Fertile eggs of White Leghorn hens were incubated in the ordinary way.

Injection of Eggs.—On the chorioallantoic membrane, unless otherwise mentioned, 0.2 cc. of 10 per cent tissue suspension in broth was injected on the chorioallantoic membrane of 7- or 8-day eggs by a modification of Burnett's method.

Intravenous.—Twelve- to 14-day eggs were used. At the position of a large vein running in the chorioallantoic membrane, a small triangular piece of shell was cut and detached from the shell membrane. Inoculum (0.05 cc.) was injected into the vein by means of a 1/4- or 1/5-mm. needle through the shell membrane which was made transparent by a small drop of cedar oil. Unless otherwise mentioned, rabbit lymph node was suspended to 10 per cent and embryo tissues to 0.5 to 1.0 per cent in 6 per cent glucose solution.

The Allantoic Sac.—Two small holes were drilled in the shell, one at the air sac end and the other at a point above the embryo. Inoculum (0.2 cc.) was injected into the allantoic sac through the second hole.

The Yolk Sac.—Five- or 6-day eggs were injected with 0.5 cc. of inoculum into the yolk sac through a small hole drilled in the shell at the air sac end.

Passage of Virus.—The method of passaging virus will be given later. As a general description, however, it seems convenient here to mention that a long-lasting effort has been made in the main strain (LA)* and in one substrain (R_1E_5) to passage the virus without preserving the materials. Exceptions to this rule were necessary only in 5 cases of the main strain, namely with chicken embryo spleens of A-E_{32}, A-E_{34}, A-E_{230}, A-E_{233}, and A-E_{237} which had been preserved in a refrigerator at −35 to −45 C. between two and ten days before they were injected into eggs of the next generation.

Reincubation of Eggs.—Unless otherwise mentioned, injected eggs were reincubated for three days at 38 C.

Test of Virus.—For a determination of virus

* Lapinized and avianized.

content either in rabbit tissues or in embryo tissues, 2 rabbits were injected intravenously with 1 cc. of tissue suspension. The result was judged as positive when the animal revealed any two of the characteristic signs of infection, *i.e.*, thermal reaction, necrotic lesions in the Peyer's patches, development of complement-fixation (c.f.) antigen in the lymph node, or c.f. antibody in the serum, or when they were resistant to subsequent challenge by intravenous inoculation with lapinized virus of certified virulence. In the later part of the study, the c.f. test which had been introduced by Nakamura and Kishi [20] for the purpose of demonstrating rinderpest antigen in infected chicken embryo spleens was often applied for the determination of infection in chicken embryos.

TABLE 1—Passage of LA Strain of Virus

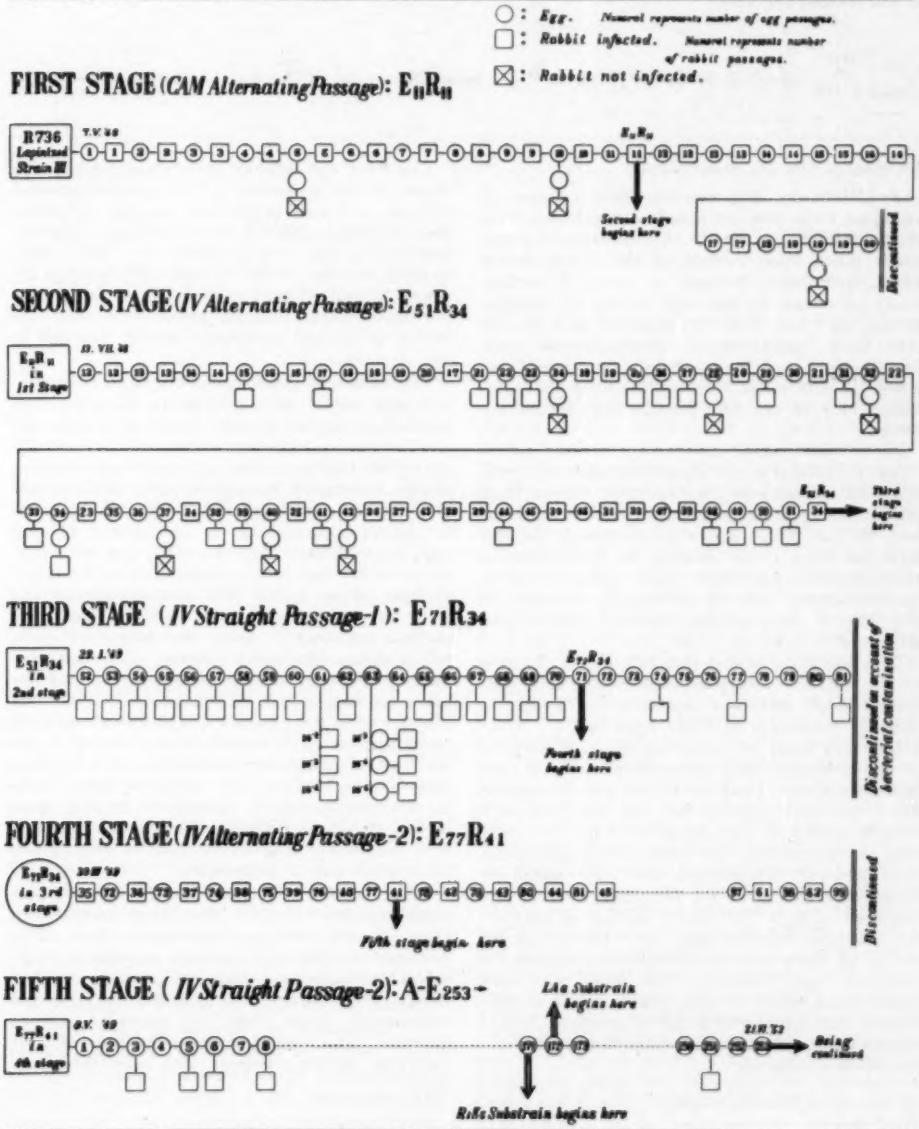
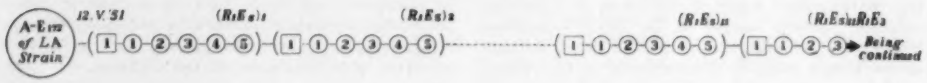

○ : Egg. Numeral represents number of egg passages.
□ : Rabbit infected. Numeral represents number of rabbit passages.
☒ : Rabbit not infected.
FIRST STAGE (CAM Alternating Passage): E11R11
R736 Lapinized Strain II
Second stage begins here
Discontinued
SECOND STAGE (IV Alternating Passage): E51R34
E11R11 in 1st Stage
Third stage begins here
THIRD STAGE (IV Straight Passage-1): E71R34
E51R34 in 2nd stage
Fourth stage begins here
Discontinued on account of bacterial contamination
FOURTH STAGE (IV Alternating Passage-2): E77R41
E77R34 in 3rd stage
Fifth stage begins here
Discontinued
FIFTH STAGE (IV Straight Passage-2): A-E253 →
E77R41 in 4th stage
LAa Substrain begins here
Being continued
Rifa Substrain begins here

TABLE 2—Passage of R_1E_2 and LA$_a$ Substrains of Virus

R_1E_2 SUBSTRAIN



LA$_a$ SUBSTRAIN

LA STRAIN AND ITS SUBSTRAINS

Preliminary Experiments.—Four cultures of lapinized virus in chorioallantoic membranes were started with 10 per cent broth suspension of lymph nodes taken from rabbits of the 733rd, 734th, 738th, and 740th passage of strain 3, respectively. Passage to the next group of embryonating eggs was made by injection with 10 per cent broth suspension of chorioallantoic membranes which had been cultured for seventy-two hours. In each series, however, the virus was maintained only in the first passage and not in the second.

First Stage (c.a.m.† Alternating Passage): E_1R_0–$E_{11}R_{11}$.—Baker,[37] in the Grosse Isle laboratory, was successful in adapting a rinderpest bovine virus for rabbits by alternating passages between calves and rabbits. Since direct serial passage of our lapinized virus, either through the intravenous or chorioallantoic membrane route, seems to be at least extremely difficult, alternating passages of this virus in embryonating eggs and rabbits were attempted in a series.

This was initiated with the 736th rabbit of strain 3. Ten per cent broth suspension of the rabbit mesenteric lymph node was inoculated on the chorioallantoic membrane of 7-day eggs (E_1R_0). After seventy-two hours of incubation, injected portions of the membranes were collected from several eggs and ground with broth in 10 per cent suspension which was then injected into the ear vein of 2 rabbits (E_1R_1) in an amount of 1 cc. for each. One of the rabbits was killed forty-eight hours after rise of temperature and the mesenteric lymph node was taken for the passage to the second eggs (E_2R_1). The similar method of alternating passages between eggs and rabbits was repeated 39 times and the passage thus reached the twentieth eggs ($E_{20}R_{19}$). This series was then discontinued, since the side series that had been started from the eleventh rabbit passage ($R_{11}E_{11}$) appeared more hopeful for adaptation of the virus for chicken embryos.

The virus was actually maintained for the full course of the series and all the rabbits injected between each egg generation revealed characteristic reactions. During these passages, however, tendency of the virus to adaptation for chicken embryos was not evidenced, since all the three attempts of two consecutive passages in chorioallantoic membranes, starting respectively with the fourth, ninth, and eighteenth rabbits, resulted in loss of virus.

The outline of LA strain is shown in table 1.

Second Stage (i.v.‡ Alternating Passage): $E_{12}R_{11}$–$E_{21}R_{24}$.—In the second stage, which was derived from the eleventh rabbit ($R_{11}E_{11}$) of the c.a.m. alternating series, the virus was intravenously transferred through chicken embryos, not always alternating with rabbit. For the passage in chicken embryos, 12- to 14 (mostly 13)-day eggs were injected intravenously, each with 0.05 cc. of 10 per cent rabbit lymph node or 0.7 to 1.0 per cent embryo spleen. The tissue suspension was made in 6 per cent glucose solution, since this medium appeared to lessen the danger of shock following an intravenous injection with the tissue. The injected eggs were incubated for seventy-two hours and then spleens were harvested for the next passage. For the passage in rabbits, the animals were injected intravenously with 1 cc. of 1 per cent embryo spleen suspension either in 6 per cent glucose or in broth, or with 0.5 cc. of rabbit blood in case two successive passages in rabbits were made. The virus materials for the next passage were obtained within twenty-four to seventy-two hours after rise of temperature.

Using rabbit injection as a check, and for protection against losing the virus, long series of embryo passages were attempted many times. This procedure resulted in 40 embryo passages with 23 rabbit passages and the last passage reached $E_{61}R_{24}$. After three zig-zag passages the virus was transferable in at least two successive embryo passages. The longest series, consisting of four successive embryo passages, were established from

† c.a.m.—chorioallantoic membrane.

‡ Intravenous.

TABLE 3—Virus Passages Through Different Routes

Initiating virus	Route of inoculation	Tissue passaged	Eggs inoculated (days)	Incubated (days)	Results of virus test (2 rabbits) No. passage 1 2 3 4
Rabbit lymph node E_4R_4, 1 stage	c.a.m.	c.a.m. 1:10	7	3	+ − + −
Rabbit lymph node E_8R_9, 1 stage	c.a.m.	c.a.m. 1:10	7	3	+ − + −
Embryo spleen $E_{10}R_{12}$, 1 stage	c.a.m.	c.a.m. 1:10	7	3	+ − + −
Rabbit lymph node $E_{31}R_{45}$, 4 stage	c.a.m.	c.a.m. 1:10	10	3-4	+ − − + − −
Embryo spleen A-E_{29}, 5 stage	c.a.m.	c.a.m 1:10, 5	9-10	3	+ − + −
Embryo spleen $E_{71}R_{24}$, 3 stage	al. cav.	embryo 1:10	7	3	− −
Embryo spleen $E_{79}R_{24}$, 3 stage	al. cav.	spleen 1:100	13-14	3-4	− − − −
Rabbit lymph node A-E_{16}-R_1, 5 stage	yolk sac	fluid 1:1	5-6	3-4	+ − − − − − − −

c.a.m.—chorioallantoic membrane; al. cav.—allantoic cavity; fluid—allantoic fluid; +—infected; −—not infected.

rabbits of $E_{29}R_{17}$, $E_{24}R_{19}$, and $E_{47}R_{23}$, but in the former two series virus was lost in the fifth passage, and in the last series its presence was not determined as all the eggs died of shock.

Two successive rabbit passages were made four times. These were, however, of no special significance, since they resulted from a lack of suitable eggs.

Third Stage (i.v. Straight Passage): $E_{52}R_{24}$–$E_{71}R_{24}$.—At this point the virus had become serially transferable through chicken embryo spleens without any rabbit passage and straight passages were now repeated 30 times. The virus was demonstrated by the rabbit inoculation in each of the early 19 passages, and since then in the twenty-third, twenty-sixth, and thirtieth passages. The series, however, could not be continued on account of bacterial contamination of the last material.

Fourth Stage (i.v. Alternating Passage): $E_{71}R_{25}$-$E_{71}R_{41}$ (=A).—When the virus was serially passaged through 20 chicken embryos in the preceding stage and reached $E_{71}R_{24}$, a side series of regular alternating passages between chicken embryos and rabbits was started as a safeguard to maintain the virus property at this point, as it was feared that limitless chicken embryo passages might result in excessive attenuation of virus either to rabbits or cattle. Though this fear was later found to be premature and the side series was discontinued after 56 alternating passages, the early part of this side series composed of 7 rabbits and 6 chicken embryos was taken up for the fourth stage and served as the bridge between two i.v. straight series of the third and fifth stages, when the former series was discontinued.

Fifth Stage (i.v. Straight Passage): A-E_1–A-E_{253}.—The straight intravenous passage through chicken embryo spleens was re-commenced from $E_{71}R_{41}$ of the fourth stage series, when the previous straight passage which had been continued in the third stage series, in parallel with the alternating passage of the fourth stage series, was discontinued. This new straight series has been continued up to date and the virus has been passaged to 253 chicken embryos without any rabbit passages. Meanwhile, no technical changes or modifications have been made except that the culture period was prolonged from three days to between three and six days, four or five days being usual in the later part of the series.

Shope and his co-workers[21] found an incubation period of seventy-two hours to be satisfactory for the culture of bovine virus on the chorioallantoic membrane. According to Nakamura and his co-workers,[20] when the lapinized virus was inoculated into the vein of the chorioallantoic membrane, the virus concentration of the embryo spleen reached the highest titer in the same culture time. Following these findings, the culture time of three days had been standard procedure from the beginning of this study. However, by the time the fifth stage began, application of complement-fixation method had attained considerable progress in demonstrating the rinderpest antigen in infected chicken embryo spleens. Results of experiments in which antigen response in this tissue was studied in detail by the c.f. method suggested that the propagation of virus in spleen tissue might often be progressive after an incubation of seventy-two hours, and even if the highest titer was reached in this period it would be maintained several more

days. The prolongation of culture time was decided after this finding.

From the beginning of this fifth stage, passage numbers are marked simply with "A-E_x", "A" standing for the last passage of the preceding stage, *i.e.*, $E_{77}R_{41}$, and "E_x" for the number of embryo passages following A. The latest passage was made on June 21, 1952, at A-E_{255} or $E_{255}R_{41}$ from the initial lapinized virus.

R_1E_5 Substrain and LA_s Substrain.—During the passage between A-E_{75} and A-E_{100} the virus attained a remarkable attenuation for cattle and became inoculable to our experimental Japanese calves without causing serious illness. Though it was unknown at that time whether or not the attenuation of virus was still progressive and whether further passages would cause the virus to lose its infectivity and accordingly its immunogenic activity to cattle, two substrains, designated with "R_1E_5" and "LA_s," were diverged from A-E_{172} and A-E_{175} respectively.

In the R_1E_5 substrain, a rabbit passage is put ahead of every five serial embryo passages, thus dividing the strain into segments of R_1E_5. Passage techniques are similar to those used in the original LA strain. Passage number is shown in the form of $(R_1E_5)_xR_1E_y$, of which $(R_1E_5)_x$ represents the number of completed segments, and R_1E_y the number of rabbit and embryo passages in the latest uncompleted segment. The present passage made on June 21, 1952, is $(R_1E_5)_{11}R_1E_5$.

As to the method of virus passage, the LA_s substrain does not differ from the original LA strain except that effort was made to reduce the times of passages by preservation of the virus at low temperature. Embryo spleens of the initiating passage A-E_{175} were collected in a small vial, frozen in dry ice-alcohol, and then preserved for forty-seven days in a refrigerator between −35 C. and −45 C. Six successive passages up to A-E_{175} were made from this preserved virus. In a similar way, A-E_{175}, A-E_{191}, A-E_{203}, and A-E_{235} were preserved for forty, sixty-three, seventy-four, and ninety-one days respectively. From preserved A-E_{235}, three successive passages were made up to A-E_{255} from which the virus was harvested on June 11, 1952, and is now under preservation. For the preserved virus,* two or three successive passages appeared to be sufficient for the recovery of its full virulence.

PROPERTIES OF THE VIRUS IN LA STRAIN

Serial Passage of the Virus in Chicken Embryos by Different Routes.—Since the beginning of the third stage ($E_{52}R_{34}$), the virus has become serially transferable through chicken embryos by intravenous inoculation with the embryo spleen. It is evident that the virus had undergone a certain adaptation for the chicken embryo during the two preceding stages of alternating passages in chicken embryos and rabbits. Now the question is whether or not the adapted virus can be serially passaged in chicken embryos by other methods. Studies have not yet covered entire levels of the passage series after the adaptation, but so far our experiments have extended up to A-E_{60}. All the attempts, *i.e.*, five chorioallantoic membrane, two allantoic cavity, and one yolk sac series, were unsuccessful. The methods and results are briefly summarized in table 3.

Pathogenicity of the Virus for Rabbits and for Chicken Embryos.—The majority of rabbits receiving the lapinized virus succumb to infection mostly within five to nine days after inoculation. Among 130 rabbits inoculated for the 100 passages of the lapinized strain 3, from the 801st to the 900th, the mortality rate during an observation period of fourteen days was about 87 per cent. The mortality among rabbits receiving the virus of LA strain has gradually decreased as seen in table 4, and it has decreased to less than 20 per cent since A-E_{61} in the fifth stage. The virus, however, still maintains the ability to cause thermal reactions and necrotic lesions in Peyer's

* The keeping quality of lyophilized virus is now being tested. The authors owe much to Capt. T. S. Grafton and to Captain Beuscher of the 406th Medical General Laboratory, U. S. Army, in Tokyo, in the lyophilization of the virus.

TABLE 4—Mortality in Inoculated Rabbits

Strain and stage		No. of passage (from–to)	Inoculated	No. of rabbits killed *	No. of rabbits died within 14 days	No. of rabbits survived	Mortality (%)
Lapinized—3		R_{801}–R_{900}	203	100	89	14	86
	1	E_1R_9–$E_{11}R_{11}$	22	12	7	3	70
	2	$E_{12}R_{11}$–$E_{51}R_{34}$	78	47	20	11	65
	3	$E_{52}R_{34}$–$E_{77}R_{34}$	38	17	14	7	67
	4	$E_{71}R_{35}$–$E_{77}R_{41}$	12	7	3	2	60
LA	5	A-E_1–A-E_{60}	74	15	28	31	48
	5	A-E_{61}–A-E_{100}	100	19	12	69	15
	5	A-E_{101}–A-E_{130}	119	13	20	86	19
	5	A-E_{131}–A-E_{200}	66	9	5	52	9
	5	A-E_{201}–A-E_{250}	22	5	1	16	6

* The animals were killed mostly within six days after inoculation for the observation of lesions and demonstration of complement-fixation antigen in the lymph node or for the harvest of lymph node virus. The number of killed animals was excluded from the calculation of mortality.

patches of the intestine, although these characteristics have become less prominent. Difficulties in clinical or postmortem diagnosis were occasionally encountered among infected rabbits. Diagnosis of such cases could be made only from results of determination of c.f. antibody response or subsequent challenge by the lapinized virus.

TABLE 5—Comparison of Spleen Weights Between Infected and Normal Chicken Embryos

Period	No. of spleens weighed	Virus inoculated	Average weight of spleens (mg.)
1	101	$A\text{-}E_{31}$–$A\text{-}E_{33}$	13.5
	159	none	8.3
2	104	$A\text{-}E_{125}$–$A\text{-}E_{135}$	12.2
	127	none	8.0
3	102	$A\text{-}E_{211}$–$A\text{-}E_{228}$	17.8
	145	none	7.0
Total	307	$A\text{-}E_{31}$–$A\text{-}E_{228}$	14.5
	431	none	7.8

Swelling and reddening of the spleen is the characteristic change in chicken embryos infected with the LA virus. This kind of change was first noticed during the passages of the second stage, but it has become a constant feature since the ninth passage of the third stage ($E_{39}R_{34}$). In the preparation of c.f. antigen, Nakamura and Kishi [28] have weighed 307 spleens collected from 18- to 19-day chicken embryos which had been inoculated with the LA virus of $A\text{-}E_{31}$ to $A\text{-}E_{228}$ at the age of 13 or 14 days, and at the same time 431 control spleens from normal chicken embryos of the same ages. The average infected spleen was nearly twice as heavy as the control; they weighed 14.5 mg. and 7.7 mg. respectively. Table 5 shows the results of estimations in three different periods of the fifth stage.

Infectivity of the Virus for Rabbits and Chicken Embryos.—According to Nakamura, Sato, and Miyamoto's estimations,[29] the rabbit minimum infective concentration (r.m.i.c.) of chicken embryo spleens which had been infected with the lapinized virus was 10^{-3} at the highest. As shown in table 6, the r.m.i.c. of chicken embryo spleens of $A\text{-}E_{30}$ and $A\text{-}E_{211}$ were 10^{-5} and 10^{-6} respectively. The possible explanation of this difference is that in the chicken embryo spleens, the LA virus propagated more prominently than the lapinized virus did, since it is extremely unnatural to presume that the LA virus was more infectious to rabbits than the lapinized virus was.

In seven experiments (table 6), two specimens of lapinized virus and five specimens of LA virus in the fifth stage were comparatively titrated in rabbits and in chicken embryos. Each of tenfold dilutions of virus material was intravenously inoculated into 2 rabbits with a dose of 1 cc., and into 5 to 7 chicken embryos with a dose of 0.05 cc. Infection in each rabbit was diagnosed in the ordinary way. The chicken embryo spleens were harvested after 4 to 6 days of incubation and pooled in groups according to the dilution of inoculated material. Infection in each group was diagnosed by inoculating 1 per cent suspension of the pooled material into 2 rabbits or in two experiments (5 and 7) by the demonstration of c.f. antigen in the pooled materials.

Results showed that m.i.c. of the lapinized virus was 1,000 to 10,000 times higher in rabbits than in chicken embryos, while m.i.c. of the LA virus was nearly comparable in both animals. The difference may be explained by the increase of infectivity to the chicken embryo or by the decrease of infectivity to the rabbit, or more probably by both as the result of chicken embryo passages of the virus.

Pathogenicity of the Virus to Cattle.—Decrease in pathogenicity of the virus for cattle has become evident after relatively many passages through chicken embryos. The first inoculation attempt in calves was conducted in the Kyushu branch of the government experimental station for animal hygiene.† Chicken embryo spleens of $A\text{-}E_{70}$ were transported there in a frozen state from our laboratory. This material was passed to 1 rabbit for the purpose of getting fresh virus. Thus, the virus inoculated into calves was $A\text{-}E_{70}\text{-}R_1$. The rabbit, revealing slight but characteristic thermal reaction, was bled on the sixth day of inoculation and

† We are greatly indebted to Dr. M. Kobayashi and Dr. K. Fukusho for conducting this experiment in their laboratory.

TABLE 6—Comparative Virus Titrations in Rabbits and Chicken Embryos

Dilution of tissue	Lap. 3 R_{557} rabbit lymph. R	Lap. 3 R_{558} rabbit lymph. R	$A\text{-}E_{30}$ embryo spleen R	$A\text{-}E_{70}\text{-}R_1$ rabbit lymph. R	$A\text{-}E_{172}$ embryo SLLKI E*	$A\text{-}E_{211}$ embryo spleen R	$A\text{-}E_{231}$ embryo SLL E*
10^{-1}		+					
10^{-2}	++	+	++	++	+−		
10^{-3}	++	++	++		pos.	++	
10^{-4}	−−	−−	++	++	pos.	++	pos.
10^{-5}	++ −−	+−	++	−−	pos.	++	+− pos.
10^{-6}	++ −−	−−	−−	−−	neg.	+−	−− neg.
10^{-7}	+−				neg.	−−	−− neg.

Lymph.—lymph node; SLLKI—spleen, lung, liver, kidney, intestine pooled; SLL—spleen, lung, liver pooled; R—rabbit; E—chicken embryo.

E*—Infection in chicken embryos was determined by demonstration of complement-fixation antigen in the spleen. +—infected; −—not infected; pos.—positive; neg.—negative.

TABLE 7—Inoculation of Calves—Experiment 1

Experiment 1, A-E_{75}–R_1—Rabbit Lymph Node, Spleen and Blood Pooled

Breed and calf No.	Dilution of virus inoculated	Incubation period (days)	Duration of fever (days)	Highest body temperature (C.)	Symptoms	Termination	Production of c.f. antibody	Reaction to challenge by bovine virus
J 112	10^{-1}	3	6	41.6	severe (diar.)	died 10d.	....	....
J 113	10^{-1}	3	6	41.8	severe (diar.)	died 9d.	....	....
J 114	10^{-2}	3	6	42.0	slight	survived	pos.	nil
J 115	10^{-2}	3	7	41.5	moderate (diar.)	survived	pos.	nil
J 116	10^{-3}	10	6	40.7	slight	survived	pos.	nil
J 117	10^{-3}	3	9	41.4	slight	survived	pos.	nil

J—Japanese black calf; diar.—diarrhea.
This experiment was conducted at the Kyushu branch of the government experiment station for animal hygiene.

the spleen and mesenteric lymph node were harvested. The solid tissues were ground in a mortar and then emulsified in 9 volumes of blood to make the virus mother suspension. Subsequent dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were made in broth and 1 cc. of each of these three dilutions was inoculated subcutaneously into 2 Japanese black calves. As shown in table 7, all the calves revealed large febrile reactions and suffered from moderate to severe symptoms of rinderpest. The 2 calves which received the 10^{-1} dilution were dead nine or ten days after inoculation. No evidence of attenuation to cattle was thus evidenced with the virus at this level of passage.

The following five experiments were carried out in our own laboratory. In experiments 2, 3, and 5, chicken embryo tissues of A-E_{160}, A-E_{175}, and A-E_{185} were tested while in experiments 4 and 6, rabbit tissues of A-E_{177}–R_1 and A-E_{188}–R_1 respectively were tested. Chicken embryo materials tested were pools of spleens, livers, lungs, kidneys, and intestines, collected from 4 or 5 chicken embryos. Rabbit materials tested were mesenteric lymph nodes of rabbits which had been infected with the passage virus of chicken embryo spleens. Since the first aim of these experiments was to learn the pathogenicity of the virus for calves, relatively concentrated tissue suspensions in broth, *i.e.*, 1 per cent in experiments 2, 3, 4, 6, and 10 per cent in experiment 5, were used at a subcutaneous dose of 1 cc. Animals inoculated were 2 Japanese black calves in experiments 2 and 4, and 2 Japanese black calves and 2 Holstein-Friesian calves in experiments 3, 5, and 6. Results are summarized in table 8. As seen in this table, all the inoculated animals, *i.e.*, 10 Japanese black calves and 6 Holstein-Friesian calves, resisted the virus without revealing any symptoms, except slight to moderate thermal reaction. The animals were not subsequently challenged by the bovine virus, since it is prohibited to use such virus in this laboratory. All the animals, however, acquired in their serum a specific c.f. antibody which was active to antigen of either bovine or lapinized strain of rinderpest virus. This antibody developed within five to thirteen days and reached the highest titer within twelve to seventeen days after inoculation. Together with 1 Holstein-Friesian control, all the calves used in experiments 5 and 6 were subsequently challenged by subcutaneous inoculation with 1 cc. of 1 per cent virulent rabbit lymph node of the lapinized virus. This inoculation was conducted in all animals at the same time, twenty-one days after the primary inoculation in the case of animals in experiment 5, and seventeen days in the case of animals in experiment 6. The animals which had been previously treated with the LA virus revealed no sign of infection, while the control calf developed a strong thermal reaction, 6 days of fever with the highest body temperature of 42.7 C., and light oral lesions; however, the animal recovered.

Between the breeds of animals, no remarkable difference in resistance to the LA virus was demonstrated in these experiments. It might be said, however, that thermal reaction and c.f. antibody response tended to be generally a little stronger in Japanese calves than in Holstein-Friesians. In table 9, temperature charts of a test calf and its control in experiment 6 are shown.

Now the LA virus in the range of passages from A-E_{160} to A-E_{188} seems to be safely inoculable in very highly susceptible cattle such as Japanese black calves. It produces sound immunity to the lapinized virus, as well as specific c.f. antibody, in either Japanese or Holstein-Friesian calves.

In February, 1952, wet and lyophilized chicken embryo spleens of A-E_{220} were sent to Dr. Reisinger at the National Institute for Veterinary Research at Pusan, Korea. It was privately learned from him that the virus had been serially passaged in rabbits and 2 Korean calves had been occasionally inoculated with it. Results obtained by him and his co-workers showed that the LA virus, up to 19 rabbit passages, was safely inoculable and strongly immunogenic to the animals, since all the 8 calves in four series receiving A-E_{220} (original specimens), A-E_{220}–R_4, A-E_{220}–R_{12}, and A-E_{220}–R_{19} respectively, revealed only slight to moderate fever reaction, no clinical symptoms, and fully resisted subsequent challenge with highly virulent bovine virus. As mentioned in the intro-

duction, Korean cattle are nearly as highly susceptible to rinderpest as Japanese cattle. Their results are believed to give strong support and good supplement to our results.

DISCUSSION

After alternating intravenous passages in chicken embryos and rabbits and subsequent straight intravenous passages in chicken embryos, the lapinized rinderpest virus has developed an evident increase in propagating property and infectivity in chicken embryos. On the contrary, a remarkable decrease in pathogenicity is shown in rabbits and cattle. Thus we were successful in producing a lapinized avianized virus. The virus and the strain are briefly designated as LA virus and LA strain respectively.

To a remarkable difference from the rinderpest virus of some bovine strains, the lapinized virus seemed to have poorer affinity to the chorioallantoic membrane of embryonating eggs. While for Shope and his co-workers, and afterwards for several other authors working with bovine strains, a number of membrane passages was the basic method to adapt the virus for chicken embryos, the same procedure, in our repeated attempts, always resulted in loss of the lapinized virus in the second passage. This feature of the virus was maintained even after 103 chicken embryo passages with 41 rabbit passages.

The LA virus has maintained, up to this time (1952), the property of strong propagation in an inoculated rabbit. It still causes characteristic thermal reaction and lesions in Peyer's patches of the intestine in this animal, though these signs of infection are not so prominent and constant as in the rabbit infected with the original lapinized virus. These features are characteristic of the LA virus and thought to constitute an important difference from the avianized virus of bovine strain origin. For the LA virus, the rabbit is not only an experimental animal but also may serve as the reactor animal yielding the vaccine material.

After 246 chicken embryo passages with 41 rabbit passages or A-E_{169}, the LA virus was attenuated to a favorable point for inoculation to highly susceptible Japanese calves. It was no longer strongly pathogenic in these calves and provoked only slight to moderate thermal reaction as a sign of infection. This grade of infectivity was maintained through A-E_{188}, and no further experiments have been conducted. Holstein-Friesians were also susceptible to

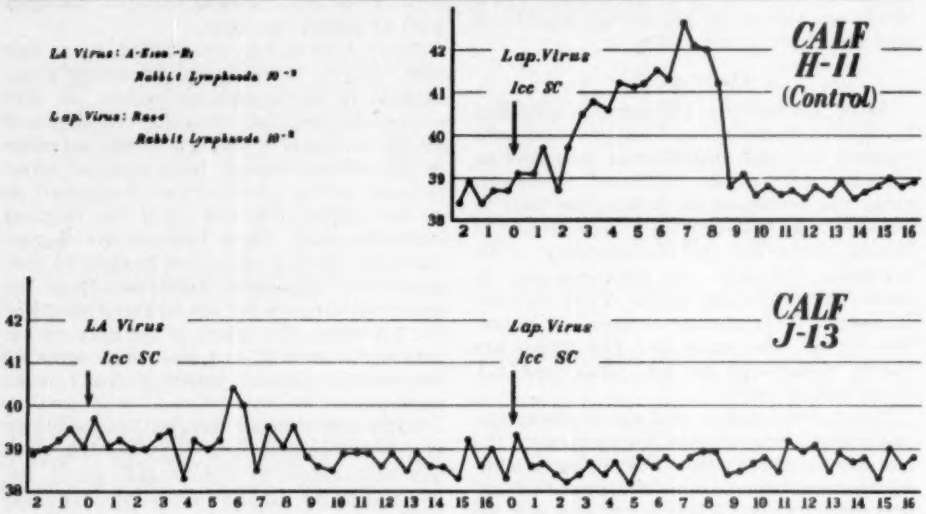
TABLE 8—Inoculation of Calves—Experiments 2, 3, 4, 5, and 6

No. of exper.	Breed and calf No.	Dilution of LA virus inoculated	Incubation period (days)	Duration of fever (days)	Highest body temperature (C.)	Other symptoms	Termination	c.f. antibody	Reaction to challenge by lapinized virus R_{324}
2	J 6	10^{-2}	4	3	40.5	nil	survived	pos.	not tested
	J 18	10^{-2}	4	3	40.6	nil	survived	pos.	not tested
3	J 1	10^{-2}	12?	2	39.9	nil	survived	pos.	not tested
	J 5	10^{-2}	5	2	41.3	nil	survived	pos.	not tested
	H 3	10^{-2}	4?	2	39.9	nil	survived	pos.	not tested
	H 6	10^{-2}	7	3	40.6	nil	survived	pos.	not tested
4	J 3	10^{-2}	4	4	40.8	nil	survived	pos.	not tested
	J 8	10^{-2}	3	4	40.6	nil	survived	pos.	not tested
5	J 10	10^{-1}	4	3	40.1	nil	survived	pos.	nil
	J 17	10^{-1}	4	7	40.6	nil	survived	pos.	nil
	H 12	10^{-1}	7	1	40.3	nil	survived	pos.	nil
	H 10	10^{-1}	5	5	40.3	nil	survived	pos.	nil
6	J 13	10^{-2}	6	1	40.4	nil	survived	pos.	nil
	J 22	10^{-2}	5	3	40.6	nil	survived	pos.	nil
	H 14	10^{-2}	8	1	40.7	nil	survived	pos.	nil
	H 13	10^{-2}	....	....	39.8	nil	survived	pos.	nil
5,6	H 11	control	....	....	....	....	.....	.....	fever 6 days (42.7 C.), oral lesions, but recovered

J—Japanese black calf; H—Holstein-Friesian calf.

Virus inoculated—exper. 2—A-E_{169} (embryo tissues pooled); exper. 3—A-E_{172} (embryo tissues pooled); exper. 4—A-E_{172}–R (rabbit lymph node); exper. 5—A-E_{188} (embryo tissues pooled); exper. 6—A-E_{188}–R (rabbit lymph node).

TABLE 9—Temperature Charts of Calves (Experiment 6)



the virus of this passage level. Though unfortunately the immunity following this virus inoculation could be challenged with the bovine virus, c.f. antibody response was demonstrated in each animal, and some animals were found firmly immune to a challenge with the lapinized virus. These findings were later enlarged and strengthened by Reisinger and his co-workers.[29] After them, the virus of A-E_{225} and rabbit passages initiating from this generation provoked similar reaction in Korean calves and produced sound immunity against challenge with the bovine virus.

The LA virus at this level of passage is remarkably less pathogenic than the lapinized virus but still infective and immunogenic in cattle of high susceptibility. Though no experiments have been made concerning contact infection from inoculated animals to susceptible ones, possibility of such events are thought to be extremely rare, since, in Nakamura and Kuroda's[6] repeated experiments, no evidence of contact infection has been obtained even with the initiating lapinized virus. Thus the LA virus is anticipated to be a safe and potent immunizing agent for highly susceptible cattle.

The lapinized virus of strain 3 maintains adequate pathogenicity and immunogenicity in cattle even after seventeen years of serial passages in more than 900 rabbits. The virus appears to have undergone little alteration of these properties since as early as the one-hundredth passage. In a remarkable contrast, Hale and Walker[24] pointed out after some chorioallantoic membrane and subsequent 58 yolk-sac passages of bovine virus a possibility that the virus may become attenuated to the point where it will no longer produce immunity in cattle. In comparison with the experiences of Grosse Isle authors and their followers, the attenuation of the lapinized virus has been induced much more slowly. During the 56 passages between A-E_{169} and A-E_{225}, distinct progress in attenuation had not been evidenced and the virus was similarly safe but infective either in Japanese or Korean calves. After how many passages the virus may become excessively attenuated can not be anticipated. The two substrains, R_1E_5 and LA_2, however, have been raised from A-E_{172} and A-E_{173}, respectively, in the expectation that one or both of these may serve as a suitable seed virus for the vaccine if and when the present strain loses its immunogenic property as the result of excessive passages.

SUMMARY

1) Initiating from the lapinized virus, the LA strain has been serially passaged through 330 chicken embryos with 41 conjunctive rabbit passages. From the difference of passage style this work is divided into five following stages:

First stage—e.a.m. alternating passage up to $E_{71}R_{71}$

Second stage—i.v. alternating passage up to $E_{31}R_{24}$

Third stage—i.v. straight passage up to $E_{71}R_{34}$

Fourth stage—i.v. alternating passage up to $E_{71}R_{71} = A$

Fifth stage—i.v. straight passage in current. The latest passage is A-E_{325} or in through number $E_{325}R_{71}$

2) During this passage, the virus has undergone a distinctive increase in infectivity for chicken embryos. The virus became directly transferable in chicken embryos after the second stage. Definite changes in infected embryo spleens have developed since $E_{60}R_{34}$ in the third stage. Relative minimum infective concentration (m.i.c.) to chicken embryos, as compared with rabbit m.i.c., has been remarkably lowered and virus specimens of the fifth stage were found infective to both chicken embryos and rabbits in nearly the same minimum concentrations.

3) The virus was gradually attenuated for rabbits in the first four stages. Mortality among rabbits has been lowered to under 20 per cent since A-E_{51} in the fifth stage. The virus still causes temperature elevation and necrotic lesions in Peyer's patches, although these reactions are less prominent and less constant than in animals inoculated with the original lapinized virus.

4) Attenuation of the virus to cattle has become evident since A-E_{169}. Japanese calves and Holstein-Friesian calves revealed slight to moderate thermal reaction but resisted without any illness an inoculation with the virus between A-E_{169} and A-E_{195}. Immune response was demonstrated by c.f. antibody test and challenge by the lapinized virus.

References

[1] Nakamura, J., Wagatuma, S., and Fukusho, K.: On the Experimental Infection with Rinderpest Virus in the Rabbit. I. Some Fundamental Experiments. J. Jap. Soc. Vet. Sci., *17*, (1938): 185-204 (Japanese original), 25-30 (English abstract).

[2] Fukusho, K., and Nakamura, J.: On the Experimental Infection with Rinderpest Virus in the Rabbit. II. Pathology. Jap. J. Vet. Sci., *2*, (1940): 75-95 (Japanese original), 96-101 (English abstract).

[3] Nakamura, J.: On the Experimental Infection with Rinderpest Virus in the Rabbit. III. Neutralization Experiment. Jap. J. Vet. Sci., *2*, (1940): 567-576 (Japanese original), 577-578 (English abstract).

[4] Nakamura, J., and Goto, Y.: On the Experimental Infection with Rinderpest Virus in the Rabbit. IV. Complement-Fixation Reaction. Jap. J. Vet. Sci., *3*, (1941): 263-282 (Japanese original), 283-286 (English abstract).

[5] Nakamura, J.: On the Experimental Infection with Rinderpest Virus in the Rabbit. V. Multiplication of the Virus in the body of the Infected Rabbit. Jap. J. Vet. Sci., *3*, (1941): 403-424 (Japanese original), 425-429 (English abstract).

[6] Nakamura, J., and Kuroda, S.: Rinderpest: On the Virulence of the Attenuated Rabbit Virus for Cattle. Jap. J. Vet. Sci., *4*, (1942): 75-98 (Japanese original), 99-102 (English abstract).

[7] Nakamura, J., Fukusho, K., and Kuroda, S.: Rinderpest: Laboratory Experiments on Immunization of Chosen Cattle by Simultaneous Inoculation with Immune Serum and Rabbit Virus. Jap. J. Vet. Sci., *5*, (1943): 455-473 (Japanese original), 474-477 (English abstract).

[8] Isogai, S.: On the Rabbit Virus Inoculation as an Active Immunization Method against Rinderpest for Mongolian Cattle. Jap. J. Vet. Sci., *6*, (1944): 371-387 (Japanese original), 388-390 (English abstract).

[9] Cheng, S. C., and Fishman, H. R.: Lapinized Rinderpest Virus. Rinderpest Vaccines, F.A.O. Agricultural Studies No. 8, United Nations, Washington, (1949): 47-65.

[10] Kuptabutra, T., Wongsongsorn, C., and Disayamaru, R.: The Use of Lapinized Rinderpest Virus in Thailand. Document presented to the F.A.O. Rinderpest Conference for Asia and the Far East at Bangkok in 1949.

[11] Alton, G. G.: Experiences in the Use of Lapinized Rinderpest Vaccine in Hongkong, 1949. Information to F.A.O. of the United Nations, Dec. 27, 1949.

[12] Fulton, A.: The Gambia, West Africa—Lapinized Rinderpest Virus Progress Report to April 18, 1949. Document presented to the F.A.O. Rinderpest Conference for Asia and the Far East at Bangkok in 1949.

[13] Reisinger, R.: Information to P.H.W. Sec., SCAP, Tokyo, April, 1950, (Letter to Lt. Col. M. W. Scothorn).

[14] Kwong, and Lee: Cited by Cheng and Fishman.

[15] Watson, H. C.: Immunization against Rinderpest with Lapinized Virus. Information to F.A.O. of the United Nations, March 15, 1951.

[16] Nakamura, J.: Not published (data lost in Inst. of Vet. Res., Pusan, Korea).

[17] Lee, N.: Private communication.

[18] Fukusho, K.: Studies on Susceptibility to Rinderpest in Japanese Black Cattle and Holsteins. 25th and 26th Meetings of Japanese Society of Veterinary Science, Jap. J. Vet. Sci., *11*, (1949): 93, and *12*, (1950): 64.

[19] Fukusho, K., and Reisinger, R.: Private communications.

[20] Nakamura, J., Sato, R., and Miyamoto, T.: Infection and Passage with Rinderpest Virus in Chicken Embryos Through Intravenous Route. Jap. J. Vet. Sci., *9*, (1947): 110.

[21] Shope, R. E., Griffith, H. J., and Jenkins, D. L.: Rinderpest: The Cultivation of Rinderpest Virus in the Developing Hen's Egg. Am. J. Vet. Res., *7*, (1946): 135-141.

[22] Shope, R. E., Maurer, F. D., Jenkins, D. L., Griffith, H. J., and Baker, J. A.: Rinderpest: Infection of the Embryos and the Fluids of Developing Hen's Eggs. Am. J. Vet. Res., *7*, (1946): 152-163.

[23] Jenkins, D. L., and Shope, R. E.: Rinderpest: The Attenuation of Rinderpest Virus for Cattle by Cultivation in Embryonating Eggs. Am. J. Vet. Res., *7*, (1946): 174-178.

[24] Hale, M. W., and Walker, R. V. L.: Rinderpest: The Production of Rinderpest Vaccine from an Attenuated Strain of Virus. Am. J. Vet. Res., *7*, (1946): 199-211.

[25] Cheng, S. C., Chow, T. C., and Fishman, H. R.: Avianized Rinderpest Vaccine in China. Rinderpest Vaccines, F.A.O. Agricultural Studies No. 8, United Nations, Washington, (1949): 29-46.

[26] Daubney, R.: Peste Bovine: Notes sur les Vaccinations par Virus Vivants. Rapport à la XIX^e Session (1951: R. No. 190).

[27] Walker, R. V. L.: Cited by Cheng and Fishman.

[28] Nakamura, J., and Kishi, S.: A Modified Method of Complement-Fixation Test in Rinderpest, 28th and 30th Meetings of Japanese Society of Veterinary Science, Jap. J. Vet. Sci., *12*, (1950): 182, and *13*, (1951): 288.

[29] Reisinger, R.: Private communication.

Investigations on the Physiological Basis for the Differential Response of Estrous and Pseudopregnant Rabbit Uteri to Induced Infection

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SEMEN collected artificially from male rabbits induces an inflammatory response of the endometrium and pyometra when introduced into uteri of pseudopregnant females.[2,7] The same semen, when introduced into uteri of estrous rabbits, produces no such gross response. That this semen is bacterially contaminated is shown by the development of a mixed colonial growth when plated on blood agar. Bacteria-free preparations of epididymal sperm, on the other hand, do not induce an inflammatory response in either estrous or pseudopregnant rabbits.[6] The action of the accessory gland secretions, absent in the epididymal "semen," has not been tested directly, but sterile preparations of cattle blood serum and mineral oil [3] have failed to induce pyometra in luteal-phase (pseudopregnant) rabbits. A direct test of the influence of bacteria was made in the present study by introducing pure cultures of various organisms into estrous and pseudopregnant rabbit uteri and observing the response.

One question of importance in the study of this inflammatory reaction was whether the differential response observed between the follicular and luteal phases of the sexual cycle was the result of a purely mechanical elimination of the irritant material from the estrous uterus by a flushing action of the uterine secretions aided by uterine motility. This was tested by ligating one cervix in estrous females and observing whether the response to an irritant differed between the freely draining uterine horn and the one from which drainage was prevented.

A further question in this study was whether the action of estrogen was responsible for the absence of the inflammatory response in the estrous uterus, or whether a high progesterone level was the causative factor in the development of pyometra in the pseudopregnant uterus. A test of the endocrine control over the uterine response was made by comparing the action of irritants in ovariectomized rabbits in the absence of, and when treated with, estrogen and progesterone, alone and in combination.

UTERINE REACTION TO MICROÖRGANISMS

Midventral laparotomies were performed in a series of estrous and ten-day pseudopregnant rabbits, and 0.2-ml. portions of suspensions of pure bacterial cultures injected into the lumens of one or both uterine horns. These suspensions were made up to a standard turbidity in order to approximate equal numbers of organisms in each inoculum. The females were destroyed twenty-four hours later and the uterine horns flushed with saline solution to recover any cellular exudate (pus) present. The washings were then centrifuged and the volume of packed debris measured.

Typical pyometra was induced in the uteri of pseudopregnant rabbits inoculated with either *Escherichia coli* or a hemolytic staphylococcus; approximately 0.4 ml. of packed debris were recovered from each such uterine horn. No lumen exudate was recovered from the uteri of estrous rabbits inoculated with these organisms.

Neither viable nor heat-inactivated (55 C. hot water bath for 30 minutes) *Vibrio fetus* organisms produced pyometra in either estrous or pseudopregnant rabbits. Microscopic examination of stained sections of the uteri of these *V. fetus*-treated uteri revealed moderate polymorphonuclear leukocytic infiltration of the endometrium of estrous females, apparently physiologically normal in animals of this reproductive state,[6] together with occasional leukocytes and some amorphous de-

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bris in the lumen. However, distinct differences between inoculated and control horns, and between those receiving live and dead inoculums, were lacking. Further, the pseudopregnant uteri revealed only slight leukocytic infiltration and cellular exudation, again without marked differentiation between treated and control horns, or live and dead *V. fetus*.

The left uterine horns of 3 additional eight-day pseudopregnant rabbits were inoculated with suspensions of viable *V. fetus* organisms; the right horns (serving as controls on the reactivity of the uteri) received portions of rabbit semen known to be bacterially contaminated. These animals were killed twenty-four, forty-eight, and 120 hours after inoculation, respectively.

After twenty-four hours' incubation, the semen had induced typical pyometra accompanied by extreme polymorphonuclear leukocytic infiltration of the endometrium and myometrium. Little further change was noted at forty-eight hours, while at 120 hours the lumen was almost free of debris and the leukocytes remaining in the endometrium were largely mononuclear. By way of contrast, *V. fetus* failed to induce more than slight leukocytic infiltration of the endometrium at any stage, and the lumen exudate (grossly visible only at 48 hours) consisted largely of an amorphous debris. As in the case of semen, there was a replacement of polymorphonuclear with mononuclear leukocytes by 120 hours.

At the time of autopsy in these pseudopregnant rabbits, a portion of each uterine horn was seared with a heated knife and a sterile pipette introduced into the lumen by puncture. Aspirated material was cultured on blood agar, nutrient broth, and other mediums to detect the presence of viable organisms. *Vibrio fetus* was recovered from all *V. fetus*-treated uteri, and a mixed bacterial growth from all semen-treated horns.

These observations suggest that an organism (e.g., *E. coli*) may be pyogenic under one set of conditions (luteal-phase uterus) and not, or at least markedly less, under another (follicular-phase uterus). *Vibrio fetus*, however, does not appear to behave in a manner similar to the more common pyogenic organisms. Further, the initial polymorphonuclear leukocytic infiltrations called forth by infective material appear to subside in a short time, to be replaced by mononuclear leukocytes usually indicative of a more chronic type of infection. In spite of this leukocytic response, infection does not appear to be eliminated readily from the luteal-phase uterus, as witnessed by the nonsterile conditions apparent five days after inoculation.

FAILURE OF UTERINE DRAINAGE AS A FACTOR IN THE INFLAMMATORY RESPONSE

The presence of a considerable leukocytic infiltration of the endometrium in estrous uteri treated with contaminated semen or microörganisms has raised the question as to why there is not an accumulation of pus in the uterus. It is known that the cervix at estrus is more relaxed than during the period when active corpora lutea are found in the ovaries. Prior observations[3] indicated that residues of sterile epididymal sperm and mineral oil were found in the luteal-phase uterus twenty-four hours after inoculation with these materials while they were absent from the estrous uterus. This suggested that constriction of the cervix and stasis of the uterine horn might be the explanation for the pyometra in the pseudopregnant rabbit.

Several trials were performed in estrous females, in which, at the time of laparotomy and inoculation of the uterus, an incision was also made through the dorsal vaginal wall exposing the right cervix. That portion of the right cervix extending into the vaginal canal was ligated by use of nylon sutures anchored in the cervical wall to prevent slippage.

In 2 such females, the inoculum administered to both ligated and control horns consisted of 0.25 ml. of ejaculated rabbit semen, diluted 1 : 4 with modified Kreb's solution. At autopsy twenty-four hours later, it was observed that both right and left horns appeared grossly normal. A small quantity of fluid, clear and free of pus, escaped from the ligated horns when opened. Otherwise, these horns appeared identical to the control horns.

Four other estrous rabbits received uterine inoculations (both horns) consisting of 0.1 ml. of dilute concentrations of *E. coli*. The right cervices were again ligated; the left horns served as controls. At autopsy twenty-four hours later, the walls of the uterine horns of 2 animals were seared with a heated knife and samples of the

lumen contents aspirated aseptically and cultured on blood agar. After several days of incubation, no growth was found in cultures of one unligated horn and only slight growth from the other unligated horn and from both ligated horns. This finding may suggest in the estrous uterus the presence of a defense mechanism highly effective against infection. The ligated uteri, when opened, revealed a highly edematous condition of the endometrium (possibly the result of trauma in ligation) and copious amounts of a watery or thin milky fluid. The left or control horns were relatively free of such material and appeared physiologically normal. Fixed and stained sections of all uteri showed an exudate consisting of amorphous material and occasional polymorphonuclear leukocytes. Appreciable numbers of leukocytes were observed infiltrating the endometrium and edema was extensive. In general, there appeared little evidence of a differential response between ligated and control inoculated uterine horns, other than the accumulation of fluids in the ligated horns.

The fluids observed in the ligated estrous uterus would probably, in the normal animal, aid in flushing foreign material and cellular exudate from the uterus through the cervix. However, failure to induce pyometra in the ligated uterus suggests some basic difference in the estrous and pseudopregnant rabbit, other than cervical drainage abetted by uterine motility. It seems not unlikely that the uterine secretions of the luteal-phase uterus, designed to nourish young embryos, may prove to be mediums also favoring rapid bacterial growth. Such conditions may increase the burden placed on the normal defense mechanisms, resulting in failure in controlling the infection. There is also the possibility that shifts in the estrogen-progesterone levels may change the uterine defense mechanisms either in character or strength.

HORMONAL CONTROL OF THE UTERINE RESPONSE

Early workers demonstrated that changes in the secondary reproductive organs in the course of the estrous cycle were under the direct control of the two hormones, estrogen and progesterone. Of these, estrogen is associated with the sexually receptive behavior at estrus, relaxation of the cervix, proliferation of the endometrium, and other functions. Progesterone, among other things, is associated with the secretory function of the uterus, receptivity of the uterus to implantation by the embryo, and the maintenance of pregnancy. The morphological and physiological changes in these organs during the estrous cycle are dependent on the relative activities of these hormones.

The differential response of the uterus, observed in the present studies, suggests a similar hormonal control. Development of pyometra in the pseudopregnant rabbit (in which corpora lutea and their secretion, progesterone, are the dominant features) might be explained on the basis of the presence of progesterone, the absence of estrogen, or a particular relationship between the two hormones.

The present study was undertaken to determine the nature of the hormonal control, if any, responsible for the striking differential behavior of the rabbit uterus to infection. For this purpose, five groups of rabbits were used: normal pseudopregnant, spayed control, spayed estrogen-treated, spayed progesterone-treated, and spayed estrogen-and-progesterone-treated. Four replicates, each consisting of 1 female of each of the five groups, were used.

The procedure followed was to perform bilateral ovariectomy (using two lumbar incisions) on 4 estrous females (day 1). Four days later (day 5), a fifth estrous female was mated to a vasectomized male to induce pseudopregnancy. The following day (day 6) hormone therapy was begun on those animals receiving treatment and continued until day 15, thus totaling ten daily injections. The pseudopregnant and 1 spayed rabbit received no treatment during this period; 1 spayed female received 2 μg. of crystalline α-estradiol (dissolved in 0.5 ml. corn oil) daily, administered subcutaneously over the shoulders; another received 1 mg. of crystalline progesterone in oil daily; and the last received 2 μg. of estradiol and 1 mg. of progesterone daily. These dosages were calculated to approximate the individual minimum estrogen and progesterone requirements of early pseudopregnancy, as postulated by Allen[3] and co-workers.

On the day of the last injection, all 5 members of the replicate were laparotomized and the right uterine horns inoculated with 0.1 ml. of the same dilute suspension of *E. coli*. The number of organisms per inoculum was later calculated from plate counts made from this suspension. The left uterine horns served as uninoculated controls. The rabbits were destroyed twenty-four hours after inoculation

and the uterine horns trimmed free from the mesometrium. One-half inch of the ovarian end of each horn was removed and fixed in formal-acetic-alcohol for histological study. The remaining portion of each horn was flushed with saline; washings were collected in tubes, centrifuged, and the volume of packed debris measured to provide some indication of quantitative response.

At the time of inoculation and again at autopsy, the gross external appearance of the uterus was noted. Spayed rabbits which received estrogen alone exhibited the characteristic sausage-like, highly contractile uteri of estrous females. Those given progesterone, alone or in conjunction with estrogen, had long, relaxed horns comparable to those of the normal pseudopregnant animal. It was noted that progesterone administered alone induced a much darker purple color in the uterus than that characteristic of pseudopregnancy.

Control horns of all animals yielded, at the most, slight traces of sediment, although some washings remained turbid after centrifugation (table 1). These traces of debris and the turbidity may be a reflection of an amorphous material (probably a secretory product of the endometrial epithelium coagulated in fixation) observed in some stained sections. Inoculated uteri of all pseudopregnant rabbits, as expected, contained pus. Inoculated uteri of the spayed control and estrogen-treated females were devoid of cellular exudate, beyond traces of an amorphous sediment. Ovariectomized rabbits treated with progesterone, alone or in combination with estrogen, generally exhibited typical pyometra. There was some suggestion that a lower grade response was obtained in those receiving both estrogen and progesterone, but the number of rabbits used (in view of the inherent variability found in biological material) was too small to provide conclusive evidence. Microscopic observations of these uteri were, in general, in close agreement with the gross findings.

The number of organisms used in inoculating each replicate varied from a low of 10,000 to a high of 3,420,000. There appears to be little correlation between the size of the infective dose and the degree of response obtained, as measured by the centrifuged exudate (table 1).

These findings point rather conclusively to progesterone as the hormone responsible, directly or indirectly, for the observed inflammatory reaction of the pseudopregnant uterus. If it is further borne out that a lesser response is obtained when progesterone and estrogen are administered simultaneously, then presumably a higher level of estrogen could override the activity of progesterone and prevent the development of pyometra in the pseudopregnant rabbit, although estrogen at this level might readily interfere with the normal course of pseudopregnancy. Conversely, a higher than normal level of progesterone at estrus might render the uterus more susceptible to the inflammatory reaction.

While a rather extensive infiltration of the endometrium by leukocytes without an appreciable exudate in the lumen appears to be associated with estrus, prolonged treatment with high levels of estrogen has been found to result in pyometra by Burrows and Kennaway [4] and Gardner and Allen [5] in the mouse, and Zondek [9] in the

TABLE 1—Inflammatory Response in Pseudopregnant and Ovariectomized Rabbits Treated with Gonadal Hormones

Type of animal	Treatment	Uterine horn	Volume of debris in uterine washings			
			Replicate 1 245,000 cells per inoculum*	Replicate 2 10,000 cells per inoculum*	Replicate 3 3,420,000 cells per inoculum*	Replicate 4 1,350,000 cells per inoculum*
Pseudo-pregnant	None	Control	Negative	Negative	Trace	Negative
		Inoculated	0.12 ml.	0.48 ml.	0.25 ml.	0.50 ml.
Spayed	None	Control	Negative	Negative	Negative	Negative
		Inoculated	Negative	Trace	Negative	Negative
Spayed	2 *gamma* estradiol**	Control	Negative	Negative	Negative	Negative
		Inoculated	Negative	Trace	Trace	Trace
Spayed	1 mg. progesterone**	Control	Negative	Trace	Negative	Negative
		Inoculated	0.45 ml.	0.05 ml.	0.40 ml.	0.50 ml.
Spayed	2 *gamma* estradiol 1 mg. progesterone**	Control	Negative	Trace	Trace	Negative
		Inoculated	0.20 ml.	Trace	0.25 ml.	0.20 ml.

* 0.1 ml. suspension of *Escherichia coli*; ** daily treatment days 6 to 15 following ovariectomy.

rabbit. That this pyometra results from infection via the cervix was shown by Weinstein, Gardner, and Allen [8] in mice in which portions of the uteri were ligated and transplanted to other regions. After estrogen therapy, the intact uterine stumps exhibited pyometra while the transplanted remnants, no longer in connection with the vagina, were normal, although distended with clear fluid. Extensive epithelial proliferation and desquamation in the cervix and vagina, resulting in occlusion of the reproductive canal and failure in normal drainage, may be responsible in part for the pyometra. Further, such accumulated debris may improve the environment for growth by existing bacteria, in turn calling forth even greater leukocytic infiltration. It then appears that the normal defensive mechanisms of the uterus at the time of estrus are conditioned by a restricted range either in estrogen level or in the ratio between estrogen and progesterone. Whether the observed leukocytes or some other agency are involved in this defense is not known.

DISCUSSION

The present studies have indicated that bacterial infection of the luteal-phase uterus may lead to inflammation of the endometrium and a pyometra comparable to that observed following insemination with ejaculated semen. However, not all organisms are pyogenic under these conditions. These observations suggest further that the pyogenicity of an organism may vary with changes in the environment in which the organism acts. Further studies will be needed to determine whether organisms or their toxic products are responsible for the results obtained here.

Histological studies of rabbit uteri indicate that a moderate leukocytic infiltration of the endometrium is characteristic of estrus. While the assumption might be made that the absence of pus and the poor recovery of bacterial inoculum in the estrous uterus are indicative of an effective defense mechanism, it is not certain that these leukocytes constitute this defense. These same polymorphonuclear leukocytes are apparently called forth in bacterial infection of the luteal-phase uterus but under these conditions fail to eliminate the infection and accumulate in the lumen as pus. It appears likely that other agencies than these leukocytes may be engaged in defense of the estrous uterus, and that these agencies are not effective in the pseudopregnant rabbit.

Normal defense in the estrous uterus may in part result from a mechanimal removal of lumen contents by a liquid exudation from the epithelium aided by uterine motility and patency of the cervix. That this is not the only mechanism involved is indicated by failure to induce pyometra in the ligated horn of an inoculated estrous uterus. Under these conditions leukocytic exudate was still scanty. This may suggest that the polymorphonuclear leukocytes seen in the subepithelial layers of the estrous endometrium do not migrate in appreciable numbers into the uterine lumen, as contrasted with the massive invasion observed in infected pseudopregnant uteri. This suggests further that there are different types of defense mechanisms operative in the two types of animals.

Whatever the actual mechanisms involved in resistance to bacterial attack, the ineffectiveness of the defensive powers of the pseudopregnant animal appears to be associated with a high effective level of progesterone. There is some indication that estrogen may be antagonistic to progesterone, but estrogen itself does not appear to be a requisite for effective defense in the estrous female since spayed individuals receiving no exogenous hormone behave in the same manner.

SUMMARY

The inflammatory response of the uterus has been studied in normal estrous and pseudopregnant rabbits following inoculation with various microörganisms. It has also been studied in estrous females in which the cervix was ligated to prevent drainage and in spayed rabbits with and without pretreatment with gonadal hormones.

The uteri of estrous and pseudopregnant rabbits were inoculated with pure cultures of two common pyogenic organisms. Pyometra was found twenty-four hours later in the luteal-phase females, while no appreciable reaction was observed in the follicular-phase uteri. *Vibrio fetus*, on the other hand, failed to induce an appreciable re-

sponse in either type of animal. These findings indicate that pyogenicity varies with changes in the tissue medium as well as with the inherent nature of the organism.

Pyometra did not develop in inoculated uteri of estrous rabbits in which the cervix had been ligated. This suggests that failure of uterine drainage is not the prime factor responsible for the accumulation of pus in the infected luteal-phase uterus.

The inflammatory response to bacterial inoculation was compared in pseudopregnant and in ovariectomized rabbits with and without hormonal treatment. Pyometra was produced in untreated pseudopregnant females and in spayed animals treated with progesterone; it was not produced in untreated or estrogen-treated ovariectomized rabbits. The response in spayed females treated with both estrogen and progesterone was less extensive than in those receiving progesterone alone. These results suggest that the normal defense mechanisms of the rabbit uterus are not dependent on the gonadal hormones; progesterone, however, appears to interfere with their efficiency.

References

[1] Allen, W. M.: Some Effects of Estrin and Progesterone in the Rabbit. Cold Spring Harbor Symposium on Quantitative Biology, *5*, (1937): 66.

[2] Black, W. G., Otto, G., and Casida, L. E.: Embryonic Mortality in Pregnancies Induced in Rabbits of Different Reproductive Stages. Endrocrinology, *49*, (1951): 237.

[3] Black, W. G., and McDonald, L. E.: Unpublished data, Univ. of Wisconsin. 1951.

[4] Burrows, H., and Kennaway, N. M.: On Some Effects Produced by Applying Oestrin to the Skin of Mice. Am. J. Cancer, *20*, (1934): 48.

[5] Gardner, W. U., and Allen, E.: Some Effects of Estrogens on the Uterus of the Mouse. Endrocrinology, *21*, (1937): 727.

[6] McDonald, L. E., Black, W. G., McNutt, S. H., and Casida, L. E.: The Response of the Rabbit Uterus to Instillation of Semen at Different Phases of the Estrous Cycle. Am. J. Vet. Res., *13*, (1952): 419.

[7] Murphree, R. L., Black, W. G., Otto, G., and Casida, L. E.: Effect of Site of Insemination upon the Fertility of Gonadotrophin-Treated Rabbits of Different Reproductive Stages. Endocrinology, *49*, (1951): 474.

[8] Weinstein, L., Gardner, W. U., and Allen, E.: Bacteriology of the Uterus with Special Reference to Estrogenic Hormones and the Problem of Pyometra. Proc. Soc. Exptl. Biol. and Med., *37*, (1936): 391.

[9] Zondek, B.: The Effect of Prolonged Application of Large Doses of Follicular Hormone on the Uterus of the Rabbit. J. Expti. Med., *63*, (1936): 789.

Cross-Agglutination Reactions Among Brucella, Vibrio, and Other Microörganisms

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LITERATURE CITED

Evans[2] reported a common antigenic component of *Brucella bronchiseptica* and *Brucella abortus*. The cross-agglutination reaction apparently is of such low magnitude that the effect is negligible. Gilman and Cameron,[5] using cattle serums of varying degrees of Brucella agglutinin titer, observed cross reactions with *Salmonella abortivoequina*, *Br. bronchiseptica*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, several Salmonella spp. and Pasteurella spp. Wong and Chow[12] demonstrated that Brucella titers developed in human subjects after *Vibrio comma* vaccinations. Eisele and associates[1] also reported that *V. comma* and Brucella spp. have common antigenic components. McCullough and co-workers[7] further demonstrated the close relationship of the cholera organism and Brucella spp. by agglutinin-absorption methods. The flagellar antigen of *V. comma* was apparently responsible for the cross reaction with Brucella antiserum. *Pasteurella tularensis* antigen may also react with Brucella antiserum and the reverse situation is also possible.[6] Felsenfeld *et al.*[4] reported that Proteus OX 19, *V. comma*, and *Salmonella pullorum* titers developed in chickens experimentally infected with brucellosis. Robertstad,[10] Simon,[11] and Morse[9] observed that *Br. abortus* and *Vibrio fetus* cross react on reciprocal agglutination tests.

The scope of this paper is the degree of cross reaction as determined by reciprocal agglutination tests conducted with Brucella spp., Vibrio spp., *S. pullorum*, and *P. tularensis* antigens using specific Brucella spp., *V. fetus*, and *P. tularensis* antiserum.

MATERIALS AND METHODS

Thirteen strains of *Brucella abortus*, six of which were adapted to grow in the absence of carbon dioxide, ten strains of *Brucella suis*, and 14 strains of *Brucella melitensis* were employed in the investigations. The strains were typical of their respective species with the exception of three strains of *Br. abortus* requiring carbon dioxide, which did not grow on either thionin or basic fuchsin agar medium plates. All the cultures were found to be of the smooth type. The strains were of diverse geographic origin and from a wide range of host species. Antiserum was produced in rabbits against *Br. melitensis*, strain M1, *Br. suis*, strain "Boar," and *Br. abortus*, U.S.D.A. antigen strain 1119-3.

Ten strains of *V. fetus* and their respective rabbit antiserums were employed in the work. Nine of the strains were of bovine origin, and one was of ovine origin. Two strains of *V. comma*, Inaba and Ogawa N.I.H. 35A5 and 41A3, respectively, were used for antigen production.

Pasteurella tularensis cells for antigen and high titer and *P. tularensis* goat antiserum were supplied to us by Dr. Lee Foshay.

Four strains of the standard type of *S. pullorum* were used. One was received from the Chicago Board of Health, one from New York State Veterinary College, and two from the Disease Control Laboratory of the Wisconsin State Department of Agriculture. In addition, the pullorum tube antigen (standard strains 7-19-20) from the Jensen Salisbury Laboratories was employed for some of the studies. The tests in which the Jen-Sal antigen was used were conducted in the same manner as the Bureau of Animal Industry method for making the serial dilutions of 1:25, 1:50, etc., in the tube test for brucellosis.

Stock cultures of *V. fetus* were maintained in tubes containing 3 to 4 ml. of Albimi Brucella broth to which agar was added to make a 0.1 per cent agar concentration. The stocks were transferred every six to eight weeks. Good growth of the transfers developed in seventy-two to ninety-six hours in an atmosphere of 10 per cent carbon dioxide at 37 C. Stock cultures were stored at room temperature. Cultures of the other bacteria used in the investigation were kept on Albimi Brucella agar slants and stored at either 4 C. (Brucella strains) or at room temperature (*V. comma* and *S. pullorum*).

The antigens were produced by growing the organisms on Albimi Brucella agar to which granulated agar was added to make a final concentration of 2 per cent. Incubation was at 37 C. for twenty-four to ninety-six hours, depending upon the species of microörganism. The *V. fetus* strains were incubated in an atmosphere of 10 per cent

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carbon dioxide in 16-oz. medicine bottles. The cultures were washed from the agar surface with 0.3 per cent formaldehyde solution containing a 0.85 per cent sodium chloride. The suspension was subsequently held in the refrigerator at 4 C. for forty-eight hours. The cell suspensions were filtered through glass wool, centrifuged, and then washed successively three times with the formalin-saline solution. The concentrated antigens were stored at 4 C. until needed. Prior to use, the antigens were standardized to a density of approximately 67 per cent light transmission in the formaldehyde-saline solution, using the Evelyn photoelectric colorimeter with the 660 μ filter. This density approximated tube 3 of the McFarland nephelometer. The *P. tularensis* cells and the pullorum tube antigen (Jen-Sal) were also washed and standardized in the same manner as the other antigens.

The agglutination tests were conducted by making a 1 : 5 dilution of the specific antiserums in the formaldehyde-saline solution. Subsequent serial dilution employed transfers of 0.5-ml. amounts of diluted serum, formaldehyde-saline solution, and antigen. In each series, the final dilutions were 1 : 10, 1 : 20, etc. The tests were incubated at 37 C. for forty-four to forty-eight hours before the readings were made. Agglutination tests, using 1-ml. amounts of the test materials, were incubated at 56 C. and read at twenty to twenty-four hours and again at forty-four to forty-eight hours. The dilution factors were the same. The results of these tests were compared with the tests incubated at 37 C. to determine the specificity of reaction according to the postulations of Feinberg and Wright.[5] Appropriate controls were included for each set of tests. In addition, the ten *V. fetus* antiserums were checked for the presence of specific Brucella globulin.[6]

Prior to the start of the investigation, the serums of the rabbits, which were employed for antiserum production, were examined for the presence of Brucella and *V. fetus* agglutinins. All were found to be negative. At this time, it was not possible to test for *V. comma*, *P. tularensis*, and *S. pullorum* agglutinins, but later agglutination testing of the serums of normal rabbits of the same colony failed to show activity against the latter antigens. The specific antiserums were stored in screw-cap vials at —20 C. until needed.

RESULTS

Brucella agglutinins were readily demonstrable in the antiserum of *V. fetus*, strain 543. The homologous agglutinin titer of this antiserum was 1 : 20,480. One strain of *Br. abortus* and four strains of *Br. melitensis* were agglutinated by a 1 : 10 dilution of the serums; four strains of *Br. abortus* and six strains of *Br. melitensis* by the 1 : 20 dilution; eight strains of *Br. abortus*, ten strains of *Br. suis*, and four strains of *Br. melitensis* at the 1 : 40 dilution. The antiserums of seven strains of *V. fetus* gave traces of agglutination with the antigens of one or more strains of Brucella. These reactions generally occurred at the 1 : 10 serum dilution, although some were observed at 1 : 40. Such results were observed more frequently with *Br. abortus* antigens. Two *V. fetus* antiserums did not react demonstrably with any of the Brucella antigens.

None of the antigens prepared from the Brucella strains were agglutinated by *V. fetus*, 543, antiserum when the tests were incubated at 56 C. for eighteen, twenty-four, forty-two, and forty-eight hours. The test of Hess and Roepke [6] for specific anti-Brucella globulin gave strongly positive results with *V. fetus*, 543, antiserum. Five *V. fetus* antiserums, I2, M2, R3, D71, and P1 gave what "appeared to be" specific reactions according to Roepke, while the other four antiserums were negative. The Hess-Roepke test and the tube-agglutination test were in agreement for seven of the ten antiserums examined. On the other hand, the former test was negative for two antiserums, while the latter was positive. The reverse situation provided the third discrepancy in the results.

In general, a considerable number of cross reactions were observed with the ten *V. fetus* antigens and the three Brucella antiserums. Four *V. fetus* strains were not agglutinated by Brucella antiserums. Incubation of the tests at 56 C. produced a decrease in Vibrio agglutinin titer of one to two tube dilutions. These results are presented in table 1.

Pasteurella tularensis cells were not agglutinated by any of the ten *V. fetus* antiserums. Agglutination of this organism was observed only with *Br. abortus* antiserum. The reaction was not observed when incubation was at 56 C. as is shown in table 1. The two strains of *V. comma*, one strain of *S. pullorum*, four strains each of *Br. abortus*, *Br. suis*, and *Br. melitensis* did not react with the *P. tularensis* antiserum.

Vibrio comma, Inaba, N.I.H., 35A5 antigen was agglutinated by the three Brucella species antiserums. The agglutinin titer was highest with *Br. suis* antiserum. *Vibrio comma* strain Ogawa, N.I.H., 41A3 gave a weak reaction with *Br. suis* antiserum but no demonstrable agglutination with *Br.*

abortus or *Br. melitensis* antiserums. The results are shown in table 1. The two strains of the cholera Vibrio were not agglutinated by the ten *V. fetus* antiserums.

The five standard *S. pullorum* strains gave agglutination cross reactions with *Br. melitensis* antiserum, three reacted with *Br. suis* antiserum, while none gave demonstrable agglutinin reactions with *Br. abortus* antiserum. Incubation of the tests at 56 C. did not consistently produce a drop in the *S. pullorum* agglutinin level as can be seen in table 1. When the *S. pullorum* antigens were tested against the ten *V. fetus* antiserums, cross reactions were observed with five *V. fetus* antiserums. The *S. pullorum* titers were not markedly depressed by incubation at 56 C. The tube test conducted with the Jensen Salsbury Laboratories' antigen employing 1 : 25, 1 : 50, etc., serial dilutions also indicated agglutination cross reactions. The results are summarized in table 2.

DISCUSSION

Agglutination cross reactions between the Brucella spp., *V. fetus*, and *S. pullorum* apparently are specific in nature. Agglutinin absorption techniques also have demonstrated [9] that the Brucella share an antigenic relationship with *V. fetus*, strain 543. Brucella share a common antigenic component with *V. fetus*, while *S. pullorum* may be related to *V. fetus* in another manner. This premise is made because *V. fetus* strain 543 antiserum contained Brucella but not *S. pullorum* agglutinins. The five *V. fetus* antiserums which agglutinated *S. pullorum* antigens did not give definite agglutination with Brucella cells. These phenomena may explain, in part, why cattle which apparently are free from vibriosis react to the Brucella serum agglutination test and vice versa. The exact significance of such reactions is not definitely known.

The observed drop in agglutinin titer following incubation of the tests at 56 C. as contrasted to 37 C. did not indicate a nonspecific type of reaction. The titer depression represented, in most instances, a single tube dilution. The least drop occurred when cross reactions were at the higher dilution levels. For these reasons, the reactions were considered to be of a specific nature.

When a phenolized saline solution (0.5 per cent phenol in a 0.85 per cent sodium

TABLE 1—Agglutination of Various Bacterial Antigens by Brucella spp. Antiserums at 37 C. and 56 C.

	Antiserums								
	Brucella abortus (1119-3)			*Brucella suis* (Boar)			*Brucella melitensis* (M1)		
	37 C.	56 C.		37 C.	56 C.		37 C.	56 C.	
Antigen	48 hr.	24 hr.	48 hr	48 hr.	24 hr.	48 hr	48 hr.	24 hr.	48 hr
Vibrio fetus I1	20	—	P10*	—	—	—	P80	P40	P40
Vibrio fetus I2	P20	P20	P20	—	—	—	P40	P40	P40
Vibrio fetus I3	—	—	—	—	—	—	—	—	—
Vibrio fetus M2	—	—	—	—	—	—	—	—	—
Vibrio fetus M3	—	—	—	P10	—	—	P20	—	—
Vibrio fetus D71	—	—	—	—	—	—	—	—	—
Vibrio fetus RL51	—	—	—	—	—	—	—	—	—
Vibrio fetus R3	20	10	P20	P80	P40	P40	P80	P20	20
Vibrio fetus P1	320	P80	P80	—	—	—	P20	P20	P20
Vibrio fetus 543	P320	P80	P80	—	—	—	P10	10	10
Salmonella pullorum N.Y.	—	....	....	10	P10	P10	P80	P40	P40
Salmonella pullorum W1	—	....	....	10	P10	P10	P20	P20	P20
Salmonella pullorum W2	—	....	....	20	P10	10	20	P20	20
Salmonella pullorum Ch	—	—	—	—	—	—	20	P10	P10
Salmonella pullorum J**	—	....	....	—	—	—	P20	P10	P20
Salmonella pullorum BAI †	—	....	....	—	—	—	P25	—	—
Vibrio comma, Inaba	P20	—	—	P80	P20	20	P20	—	—
Vibrio comma, Ogawa	—	—	—	P10	—	—	—	....	....
Pasteurella tularensis	P20	—	—	—	....	....	—	....	....
Brucella abortus (1119-3)	5120	P1280	P2560	....	....	....	....	....	....
Brucella suis	....	....	....	P10,240	2,560	P5,120	....	....	....
Brucella melitensis (M1)	....	....	....	....	....	....	P5,120	1,280	P2,560

* P = Incomplete or partial agglutination of the antigen at the given dilution.

** Jen-Sal antigen resuspended in the formaldehyde-saline solution and this saline diluent employed throughout the test series.

† Jen-Sal antigen employing the B.A.I. method of making serial dilutions as in the tube test for brucellosis.

— = No demonstrable agglutination.

TABLE 2—Agglutination of Salmonella Pullorum Antigens by the Antiserums of Five Strains of Vibrio Fetus at 37 C. and 56 C.

Antigen	*Vibrio fetus* Antiseram*															
	I1				I3				M2				RL51				D71			
	37 C.		56 C.		37 C.		56 C.		37 C.		56 C.		37 C.		56 C.		37 C.		56 C.	
	48 hr.	20 hr.	48 hr.		48 hr.	20 hr.	48 hr.		48 hr.	20 hr.	48 hr.		48 hr.	20 hr.	48 hr.		48 hr.	20 hr.	48 hr.	
S. pullorum NY	P160‡	P20	P40		P80	P40	P80		P80	P40	P80		P320	P160	P320		P160	P40	P40	
S. pullorum W1	P40	P40	P40		P40	P20	P40		80	P40	P40		320	P320	P320		P80	P40	P40	
S. pullorum W2	P80	P40	P40		160	P80	P160		80	P40	P80		160	P160	P160		P80	P40	P40	
S. pullorum Ch	P40	P10	P10		P40	P20	20		P40	P20	P20		80	P80	P80		P40	P10	P10	
S. pullorum J**	P20	P20	P20		P20	P20	20		P40	P20	P20		160	P40	P160		40	P10	P20	
S. pullorum J† (B.A.I. method)	P25	—	—		P50	P25	P25		P50	P25	P25		P50	P25	P25		P50	P25	P25	
Homologous *V. fetus* antigens	P5120	P5120	P5120		P5120	640	P5120		P20,480	20,480	P20,480		P20,480	20,480	P20,480		P20,480	P20,480	P20,480	

* Cross reactions were not observed between the 6 *S. pullorum* antigens and 5 *V. fetus* antiserums (12, M3, 543, P1, and R3).

** Jen-Sal antigen resuspended in formaldehyde-saline solution and this saline diluent employed throughout the test series.

† Jen-Sal antigen employing the B.A.I. method of making serial dilutions as in the tube test for brucellosis.

‡ P = Incomplete or partial agglutination of the antigen at the given dilution.

— = No demonstrable agglutination.

chloride solution) was employed as a diluent and used to suspend the *S. pullorum* cells, the cross reactions with the Brucella and *V. fetus* antiserums were still evident.

The fact that a number of genera and species may share one or more common antigenic components presents some problems for investigation in the field of diagnostic serology. It is possible that a number of the Salmonella share a relationship with these several organisms and that the demonstration of such a common factor or factors may be useful in Salmonella typing procedures.

Summary

It was determined that *Salmonella pullorum, Vibrio fetus,* and Brucella spp. are antigenically related. The effect of this relationship upon the various agglutination diagnostic tests is unknown. *Pasteurella tularensis* and *Vibrio comma* share antigenic components with the Brucella spp. but not with *V. fetus.* Investigations are being conducted to elucidate various phases of this problem.

References

[1] Eisele, C. W., McCullough, N. B., Beal, G. A., and Burrows, W.: Development of Brucella Agglutinins in Humans Following Vaccination for Cholera. Proc. Soc. Exptl. Biol. and Med., *61,* (1946): 89-91.

[2] Evans, A.: Further Studies of *Bacterium Abortus* and Related Bacteria. II. A Comparison of *Bacterium Abortus* with *Bacterium Bronchisepticus.* J. Infect. Dis., *22,* (1918): 580-593.

[3] Feinberg, R. J., and Wright, G. G.: Factors Influencing the Agglutination Titration in Human Brucellosis. J. Immunol., *67,* (1951): 115-122.

[4] Felsenfeld, O., Young, V. M., Loeffler, E., Ishihara, S. J., and Schroeder, W. F.: A Study of the Nature of Brucellosis in Chickens. Am. J. Vet. Res., *12,* (1951): 48-54.

[5] Gilman, H. L., and Cameron, H. S.: The significance of Low Agglutination Titers in Bang's Disease. Cornell Vet., *26,* (1936): 113-119.

[6] Hess, W. R., and Roepke, M. H.: A Nonspecific Agglutinating Substance in Bovine Serum. Proc. Expti. Biol. and Med., *77,* (1951): 469-472.

[7] McCullough, N. B., Eisele, C. W., and Beal, G. A.: Antigenic Relationship of Brucella and *Vibrio comma.* J. Infect. Dis., *83,* (1948): 55-59.

[8] McCullough, N. B.: Laboratory Tests in the Diagnosis of Brucellosis. Am. J. Pub. Health, *39,* (1949): 866-869.

[9] Morse, E. V.: Unpublished data. 1951.

[10] Robertstad, G. W.: Serological Relationship of *Vibrio Fetus.* Master of Science Thesis, University of Wisconsin Graduate School, 1951.

[11] Simon, J.: Histopathological Alterations of the Bovine Uterus. Doctor of Philosophy Thesis, University of Wisconsin Graduate School, 1951.

[12] Wong, D. H., and Chow, C. H.: Group Agglutinins of *Brucella abortus* and *Vibrio cholerae.* Chinese M. J., *52,* (1937): 591. Cited by I. F. Huddleson: Brucellosis in Man and Animals. Commonwealth Fund, New York, 1943.

The Effect of Dielectric Heat on Anthrax Spores Under Feed Bag Processing Plant Conditions

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RECENT outbreaks of anthrax in the Middlewest have necessitated the reëxamination of the procedures employed by feed manufacturers in sanitizing used feed bags. In the present crisis, Illinois has promulgated a regulation permitting the use of new burlap, cotton, or paper bags, or cleaned and sterilized, or fumigated, burlap or cotton bags. The use of new bags only would increase materially the cost of the 100-lb. bag which carries the feed from the feed-processing plant to the farmer. Feed container cost can be greatly reduced by having the bags make five to seven trips from mill to farm. However, it is of utmost importance that all disease-producing organisms on the bags be destroyed between trips.

It has long been known that heat, and heat alone, is one of the most satisfactory means of destroying disease-producing organisms. Jungherr [1] reported dielectric heat as "capable of effectively killing all microbiologic agents of potential disease-making power except spore-forming organisms." Jungherr's experiments were carried out under pilot plant or experimental conditions at the General Electric Com-

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Fig. 1—Dielectric heat ovens employed in the experiments. Each oven is over 66 ft. long, 14 ft. high, and about 12 ft. wide. The metal link-chain belt acts as a negative pole and moves the bales through the oven at the rate of about 5 in. per minute. The oven contains three dielectric units, each using 40,000 watts electricity per hour at 10,000 volts.

pany plant, Schenectady, N. Y. He subjected test strips of contaminated burlap, placed in the middle of a 34-bag experimental bale, to 93 to 138 C. dielectric heat for five to nine minutes. *Bacillus anthracis*, the causative agent of anthrax, is an aërobic spore-forming organism and, thereby, would presumably not be killed under the pilot plant conditions. It is the purpose of this paper to report the effect of dielectric heat on anthrax spores when handled under actual feed bag processing plant conditions.

Materials

The dielectric ovens employed in this investigation were constructed by the General Electric Company, Schenectady, N. Y., for the Eastern States Milling Company, Buffalo (fig. 1). Flach[2] described the apparatus which employs the principle of diathermic physiotherapy. Heat results from the resistance encountered by passing rapidly reversing (14 million times a second, 10 thousand volts) electric currents through the bale of burlap bags. Almost instantaneous and uniform heat penetration occurs. The bale of 250 bags is just as hot in the center as on the outside. Temperatures in the bales are checked with a needle pyrometer.

Employing a culture of *B. anthracis* isolated from imported bone meal, contaminated burlap and cotton test strips were prepared in the laboratory. A seven- to ten-day old growth of the culture was washed from agar slants and used to prepare a standardized suspension of cells. The density of the suspension was adjusted to tube 5 of the McFarland nephelometer, using the Lumetron photoelectric colorimeter. Microscopic examination of Gram- and spore-stained smears revealed approximately 50 per cent of the cells to be in the spore stage. Inch-square strips of the burlap and cotton material were contaminated with 0.5 ml. of the cell suspension and placed in glass shell vials or wrapped individually in a paper packet.

For the first trial, contaminated burlap and cotton test strips were placed in sterile cork-stoppered glass shell vials. Brown wrapping paper was tied around the corks.

In the second trial, only burlap test strips were used. Each strip was contaminated, air dried, and wrapped in a single thickness of brown wrapping paper.

Bacteriological examinations were conducted on the cotton and burlap strips to determine the viability of the spores. Each strip was removed from the container and suspended in a tube containing approximately 5 ml. of sterile brain heart infusion broth. After shaking, subcultures of the broth were made immediately onto brain heart infusion agar plates. Subcultures were also made after incubating the broth tube for twenty-four hours at 37.5 C. Selected colonies on the pre- and postincubation subculture plates were picked for identification. Typical anthrax colonies displayed the medusa head appearance, were nonmotile in semisolid agar, and killed either mice or guinea pigs.

TABLE 1—Exposure of Anthrax Spore Contaminated Burlap and Cotton Strips to Dielectric Heat

Bale (No.)	Temperature and holding time	Results
1	127 C., ½ hour	Anthrax spores —
2	127 C., 1½ hours	Anthrax spores —
3	127 C., 2½ hours	Anthrax spores —
4	123.9 C., 3½ hours	Anthrax spores —
5	146.1 C., 1 hour	Anthrax spores —
6	110 C., ½ hour	Anthrax spores +
Laboratory held controls		Anthrax spores +
Shipped controls		Anthrax spores +

— No viable bacteria isolated; + viable bacteria isolated.

Procedure and Experimental Results

In the first trial, five vials containing burlap strips and five vials containing cotton strips were placed in the upper half of each of six bales of bags. The bales, each containing 250 bags, were then exposed to dielectric heat, brought to the desired temperature, and held at that temperature for varying periods of time. The unopened vials were returned to the laboratory for bacteriological examination in order to determine the viability of the spores (table 1).

In the second trial, 15 burlap strips were placed in the upper half of each of four bales of bags. The individual bales were weighed before and after heating in an effort to determine the weight loss of the bales through evaporation of moisture, volatile oils, etc., during the heating process. The unopened wrapped strips were returned to the laboratory for bacteriological examination (table 2).

Discussion

Under the experimental conditions of the first trial, *B. anthracis* spores were apparently killed when subjected to dielectric heat for one half hour or longer at the temperatures of 127 C. and 146.1 C. The spores were not all destroyed when exposed to 110 C. for one half hour. In this trial, the burlap and cotton strips were quite moist and in glass containers. Neither condition simulated plant conditions; however, the results warranted further investigation.

The procedure employed for heat treating the spores in the second trial more nearly coincided with actual plant conditions. The contaminated strips contained approximately the same amount of moisture (8.5 %) as was present in the baled

TABLE 2—Exposure of Anthrax Spore-Contaminated Burlap Strips to Dielectric Heat

Bale (No.)	Temperature and holding time	Weight of bale before heating (lb.)	Weight of bale after heating (lb.)	Weight loss (%)	Results
1	112.7 C., ½ hour	224.0	219.5	2.01	Anthrax spores —
2	121 C., ½ hour	226.0	214.0	5.31	Anthrax spores —
3	129.4 C., ½ hour	224.5	211.5	5.79	Anthrax spores —
4	132 C., ½ hour	222.5	211.0	5.17	Anthrax spores —
Laboratory held controls					Anthrax spores +
Shipped controls					Anthrax spores +

— No viable bacteria isolated; + viable bacteria isolated.

burlap bags. The single thickness of wrapping paper, necessary to prevent escape of viable spores in the feed bag plant, was the only intervening factor at odds with simulating plant conditions.

Under the conditions of the second trial, *B. anthracis* spores were apparently killed when subjected to dielectric heat for one half hour at temperatures of 112.7 C., 121 C., and 132 C. The weight loss in this operation revealed a 2.01 per cent loss when the bale was heated to 112.7 C. and held at that temperature for one half hour, 5.31 per cent at 121 C., 5.79 per cent at 129.4 C., and 5.17 per cent at 132 C.

Murray [3] reported evidence, based on extensive and well-controlled experiments, indicating that a temperature of 135 C. for five to ten minutes was necessary to kill anthrax spores in the absence of moisture whereas, under moist conditions, spores suspended in normal (0.8 %) saline solution were killed in five to ten minutes at a temperature of 100 C. Since the anthrax spores exposed to dielectric heat under plant conditions were apparently destroyed at a temperature lower than that quoted for dry heat, it was assumed that the hot moisture released from the burlap bags had a definite lethal effect on the spores.

The increased moist heat effect obtained in the dielectric ovens installed at Buffalo, as compared with the pilot plant ovens at Schenectady, was credited with the superior results obtained by us in the dielectric heat destruction of anthrax spores. Burlap normally contains about 8.5 per cent moisture. The increased moist heat was attributed to the longer holding temperatures maintained and to the larger size bales (250 bags per bale as compared to 34 bags per bale) employed, thus providing more available moisture.

SUMMARY

The necessity of killing *Bacillus anthracis* spores in used feed bags was emphasized by recent outbreaks of anthrax in the Middlewest.

Two experiments were conducted under actual feed bag plant-processing conditions, with dielectric heat treatment of burlap strips artificially contaminated with anthrax spores. The spores were killed at temperatures of 112.7 C. and above when the temperatures were maintained for at least one half hour.

Moisture content in bales of burlap bags may presumably vary and influence the results of the dielectric heat treatment. In these tests, the burlap bags contained approximately 8.5 per cent moisture, which was considered normal. On the basis of two separate exposures of anthrax spores to dielectric heat, it appears that this method of sanitizing bags may be useful in destroying anthrax spores. Further observations would seem desirable before definite conclusions are drawn.

References

[1] Jungherr, Erwin: Studies on Sanitizing Used Feed Bags. J.A.V.M.A., *117*, (1950): 324-328.

[2] Flach, Ward: New Method Will Sanitize Eastern States Feed Bags. Eastern States Coöperator, Eastern States Farmers' Exchange, West Springfield, Mass., *25*, (Dec., 1949): 5-6.

[3] Murray, T. J.: Thermal Death Point. II. Spores of *Bacillus anthracis*. J. of Infect. Dis., *48I*, (1931): 457-467.

Effect of Pseudorabies Virus (Aujeszky Strain) in the Large Brown Bat *(Eptesicus Fuscus)*

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THE AUJESZKY strain of pseudorabies virus was originally isolated in Hungary [1] and is identical with the pseudorabies (mad itch) virus causing disease in the United States. Hurst [2] found rabbits susceptible to this virus when inoculated by peripheral routes. Susceptible hosts such as cattle, cats, and dogs die following inoculation by any route. The present study was undertaken to determine whether the large brown bat *(Eptesicus fuscus)* was susceptible to the virus by various routes of inoculation.

METHODS AND RESULTS

The virus employed in this study was the Aujeszky strain of pseudorabies virus furnished by the American Type Culture Collection in Washington, D. C. This virus carried in infected rabbit brain had originally been obtained from A. Aujeszky in 1931. Before receipt at this laboratory, the virus had been passed several times in rabbits. The exact number of passages in rabbits is unknown. The virus-bearing rabbit brain was suspended in glycerine when received at this laboratory and was ground in a mortar with alundum and diluted to a 20 per cent suspension with physiological saline. This 20 per cent suspension was used as inoculum throughout the present study.

Prior to inoculation of the bats, 2 guinea pigs were injected intradermally with 0.1 cc. of the inoculum to determine whether typical symptoms of pseudorabies could be produced with the present strain of virus. After three days, the guinea pigs showed characteristic symptoms such as scratching and biting of the skin at the site of inoculation, excessive salivation, and prostration, followed by death.

Before initiating the present experiment, 36 bats (age unknown) were kept in the refrigerator at 40 F. for one week. These bats were then divided into 12 groups of 3 bats each. Three bats from each group, except for one group of controls, were administered the virus by one of the following methods: intracerebral, intraperitoneal, intradermal, intramuscular, intralingual, intraocular, intratesticular, and intracardiac injection, and oral, rectal, and intranasal instillation. Three-hundredths cubic centimeter of the above inoculum was given to the bats in the intracerebral, rectal, and intranasal groups. The animals in each of the remaining groups were inoculated with 0.6 cc. of the suspension. After exposure to the virus by the above mentioned routes, all groups were observed twice daily for symptoms characteristically found in mammals infected with pseudorabies virus.

Between seventy-two and ninety-six hours all bats displayed nervous symptoms, grinding of the teeth, salivation, followed by prostration, and death. As these symptoms appeared, the bat was destroyed and the brain removed aseptically and stored in a CO_2 deep-freeze storage vault. After all bats were destroyed, the brains were thawed, and those from each group were pooled and ground to a 20 per cent suspension with physiological saline. The 3 bats in the control group appeared normal and were discarded.

Brain suspension material taken from the bats inoculated with the virus was shown to contain pseudorabies virus by the following procedure: unvaccinated guinea pigs and guinea pigs previously immunized against pseudorabies virus were injected intradermally with the above suspensions. Immunization had been accomplished by intradermal injection of high dilutions of the original inoculum over a period of six weeks. After three to six days the unvaccinated guinea pigs showed typical symptoms of pseudorabies. The immunized guinea pigs remained normal during the fourteen-day observation period.

TABLE 1—Response of the Large Brown Bat (Eptesicus Fuscus) to Pseudorabies Virus (Aujeszky Strain)

Route of inoculation	Bats exposed (No.)	Showing symptoms (No.)	*Min. to max. incubation period (hours)
Intraperitoneal	3	3	72 – 96
Rectal	3	3	.....
Intradermal	3	3	.....
Intracardiac	3	3	.....
Intramuscular	3	3	.....
Intralingual	3	3	.....
Intraocular	3	3	.....
Intratesticular	3	3	.....
Intranasal	3	3	.....
Oral	3	3	.....
Intracerebral	3	3	.....
Normal controls	0	0	0

* Incubating period = time between exposure to virus and onset of symptoms.

From the Live Stock Sanitary Service Laboratory, Maryland State Board of Agriculture, University of Maryland, College Park.

Appreciation is expressed for the technical assistance of Suzanne Moore.

DISCUSSION

Table 1 shows the response of the large brown bat *(Eptesicus fuscus)* to exposure of pseudorabies virus by various routes. The incubation period in bats was between seventy-two and ninety-six hours. The bats showed no scratching and biting of the skin as is usually found in guinea pigs, mice, rabbits, and hamsters exposed to this virus but did show extreme, nervous tremors, salivation, and grinding of their teeth followed by prostration and death.

SUMMARY

A strain of pseudorabies virus (Aujeszky strain) isolated in Hungary by Aujeszky and passaged several times in rabbits (exact number of passages unknown) has been successfully transmitted to the large brown bat *(Eptesicus fuscus)* by the following routes of exposure: intracerebral, intradermal, intraperitoneal, intramuscular, intracardiac, intraocular, intralingual, intratesticular, oral, nasal, and rectal. The normal bats not exposed to the virus appeared normal throughout the duration of this experiment. The virus contained in the brains of bats showing nervous tremors and bats succumbing to this disease was confirmed to be pseudorabies by intradermal injection into unvaccinated guinea pigs and into guinea pigs vaccinated against pseudorabies virus. The vaccinated guinea pigs showed no pseudorabies symptoms, while the unvaccinated guinea pigs showed symptoms characteristically found in animals infected experimentally by intradermal injection with pseudorabies virus.

References

[1] Aujeszky, A.: Ueber eine Neue Infektionskrankheit bei Haustieren. Cent. Bakt. I, Abt. Orig., *32*, (1902): 353-357.

[2] Hurst, E. W.: Studies on Pseudorabies (Infectious Bulbar Paralysis, Mad Itch). II. Routes of Infection in the Rabbit, with Remarks on the Relation of the Virus to Other Viruses Affecting the Nervous System. J. Exptl. Med., *59*, (1934): 729-749.

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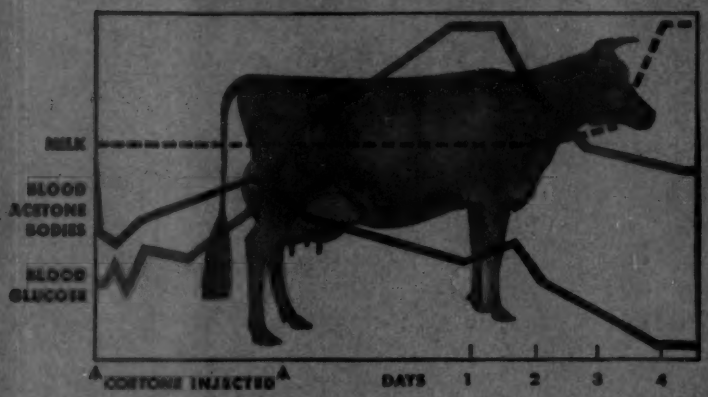

For Dramatic Relief of Bovine KETOSIS

A single intramuscular injection of CORTONE Acetate *Veterinary* often effects complete remission of symptoms in both pre- and postpartum ketosis of dairy cows, in many instances after other forms of therapy have failed. Blood sugar levels have returned to normal in twenty-four hours, and milk production has been restored to normal in as little as three days.

Paralleling the dramatic results achieved by CORTONE in human medicine, CORTONE Acetate *Veterinary* also has been found effective against rheumatoid arthritis and inflammatory conditions of the eyes and skin. Its field of usefulness in animals continues to broaden as research progresses.

CORTONE Acetate Veterinary is available through a number of veterinary pharmaceutical manufacturers, in addition to pharmacies. Use is restricted to the veterinary profession.

CORTONE is the registered trade-mark of Merck & Co., Inc. for its brand of cortisone.

MERCK & CO., INC.

Manufacturing Chemists

RAHWAY, NEW JERSEY

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